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Blood phenylalanine reduction corrects CNS dopamine and serotonin deficiencies and partially improves behavioral performance in adult phenylketonuric mice

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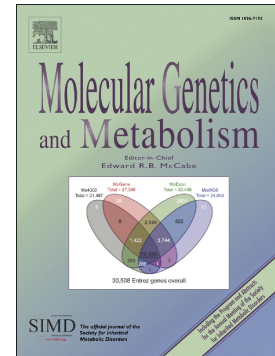
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ABSTRACT

Central nervous system (CNS) deficiencies of the monoamine neurotransmitters dopamine and serotonin have been implicated in the pathophysiology of neuropsychiatric dysfunction in human phenylketonuria (PKU). In this study, we confirmed the occurrence of brain dopamine and serotonin deficiencies in association with severe behavioral alterations and cognitive impairments in hyperphenylalaninemic C57BL/6-*Pah*^{enu2/enu2} mice, a model of human PKU. Phenylalanine-reducing treatments, including either dietary phenylalanine restriction or liver-directed gene therapy, initiated during adulthood were associated with increased brain monoamine content along with improvements in nesting behavior but without a change in the severe cognitive deficits exhibited by these mice. At euthanasia, there was in *Pah*^{enu2/enu2} brain a significant reduction in the protein abundance and maximally stimulated activities of tyrosine hydroxylase (TH) and tryptophan hydroxylase 2 (TPH2), the rate limiting enzymes catalyzing neuronal dopamine and serotonin synthesis respectively, in comparison to levels seen in wild type brain. Phenylalanine-reducing treatments initiated during adulthood did not affect brain TH or TPH2 content or maximal activity. Despite this apparent fixed deficit in striatal TH and TPH2 activities, initiation of phenylalanine-reducing treatments yielded substantial correction of brain monoamine neurotransmitter content, suggesting that phenylalanine-mediated competitive inhibition of already constitutively reduced TH and TPH2 activities is the primary cause of brain monoamine deficiency in *Pah*^{enu2} mouse brain. We propose that CNS monoamine deficiency may be the cause of the partially reversible adverse behavioral effects associated with chronic HPA in *Pah*^{enu2} mice, but that phenylalanine-reducing treatments initiated during adulthood are unable to correct the neuropathology and attendant cognitive deficits that develop during juvenile life in late-treated *Pah*^{enu2/enu2} mice.

Keywords:

Phenylketonuria, phenylalanine hydroxylase, tyrosine, tryptophan, dopamine, serotonin, tyrosine hydroxylase, tryptophan hydroxylase, cognition, behavior

Abbreviations:

PKU – phenylketonuria, PAH – phenylalanine hydroxylase, Phe – L-phenylalanine, Tyr – L-tyrosine, Trp – L-tryptophan, LNAA – large neutral amino acids, L-DOPA – L-3,4-dihydroxyphenylalanine, DA – dopamine, HVA – homovanillic acid, 5-HTP – L-5-hydroxytryptophan, 5-HIAA – 5-hydroxyindoleacetic acid

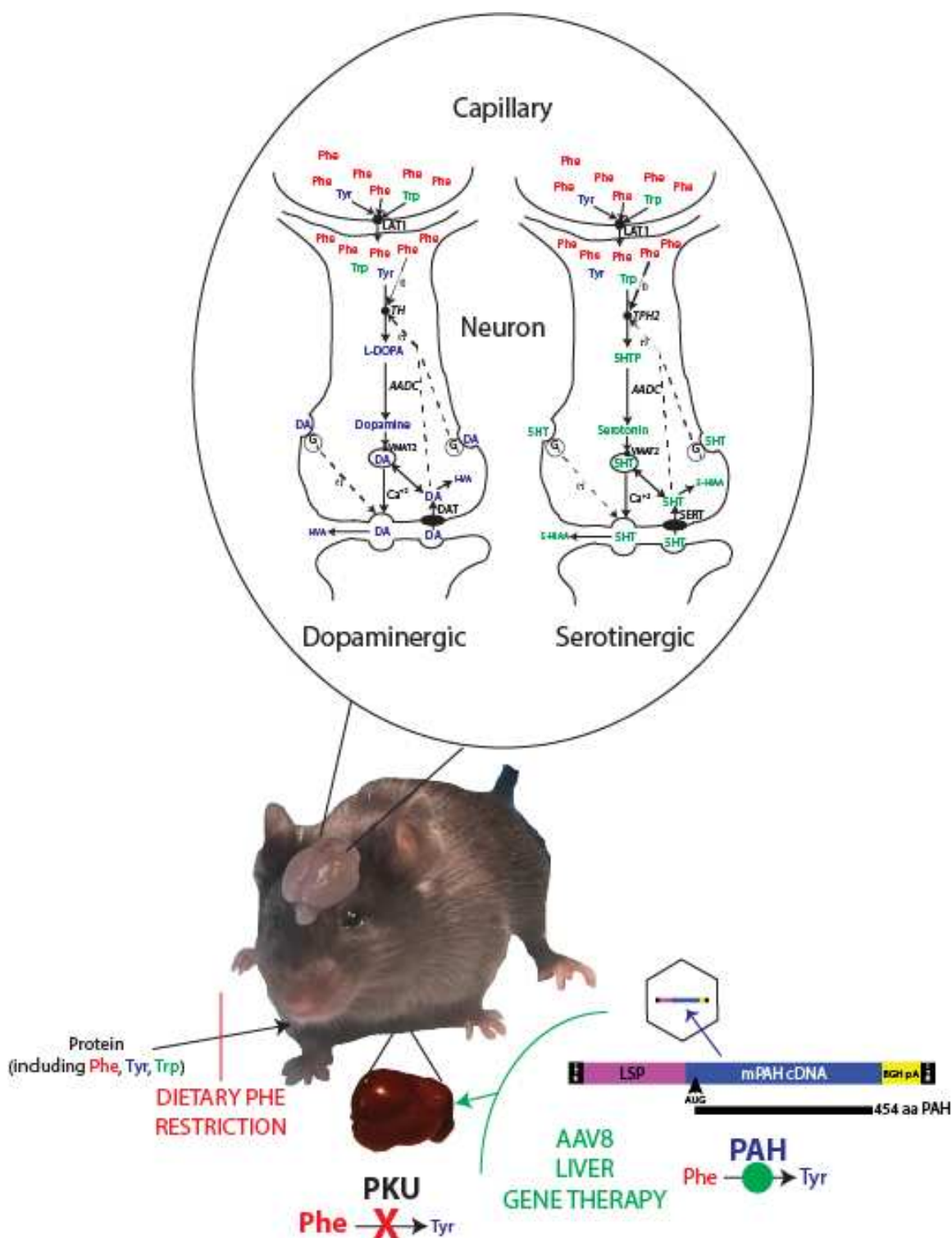
1. INTRODUCTION

Phenylketonuria (PKU), due to recessively inherited phenylalanine hydroxylase (PAH) deficiency (**Figure 1**) (OMIM # 261600), is among the most common inborn errors of metabolism in humans. Hyperphenylalaninemia (HPA), if left untreated, is associated with severely impaired brain growth and development yielding profound cognitive disability. Early disease detection through neonatal screening and initiation of dietary phenylalanine (Phe) restriction within the first weeks of life prevents the major manifestations of the disease (Azen et al. 1991). However, chronically elevated blood Phe due to loss of dietary control in adolescents and adults with early-treated PKU is frequently associated with disturbed executive functioning (VanZutphen et al. 2007; Christ et al. 2010), psychiatric symptoms (Bilder et al. 2013), and in extreme cases can be associated with adult onset white matter degeneration, ataxia and seizures (Thompson et al. 1990). The proximate pathologic mechanisms that mediate the neurobehavioral effects of HPA are incompletely understood, but abundant evidence implicates dysfunction of the dopaminergic and serotonergic neuronal systems as having major roles in the symptoms of anxiety and impaired executive functioning associated with HPA (Christ et al. 2010; de Groot et al. 2010; Feillet et al. 2010). Here, we further investigate the relationships between HPA, monoamine neurotransmitter deficiency, behavior, and cognition in a murine model of human PKU.

The *Pah*^{enu2} mouse, a model of human PKU (McDonald et al. 1990), harbors a missense mutation in the seventh exon of the *Pah* gene (McDonald and Charlton 1997) that yields complete PAH deficiency in homozygous mice. Untreated *Pah*^{enu2/enu2} mice exhibit HPA, growth failure (McDonald 2000), hypopigmentation, maternal effects upon fetal development (McDonald et al. 1997) and cognitive deficits including impaired memory (Zagreda et al. 1999; Cerreto et al. 2012; Bruinenberg et al. 2016) and spatial recognition (Cabib et al. 2003). Significant CNS dopamine and serotonin deficiencies have been measured in hyperphenylalaninemic *Pah*^{enu2/enu2} mice (Puglisi-Allegra et al. 2000; Pascucci et al. 2002; Pascucci et al. 2008; Harding et al. 2014; Winn et al. 2016), and these deficiencies have been implicated in the pathogenesis of the memory deficits exhibited by these animals (Zagreda et al. 1999; Bruinenberg et al. 2016). The central

elements of monoamine neurotransmitter synthesis and the turnover of these neurotransmitters are depicted in **Figure 1** and inspection of the pathway leads to the two hypotheses proposed to explain monoamine neurotransmitter deficiency in PKU. According to the first hypothesis, HPA competitively reduces transport of L-tyrosine (Tyr) and L-tryptophan (Trp), the substrates for dopamine and serotonin synthesis respectively, across the blood-brain barrier and into the brain (Pietz et al. 1999). According to the second hypothesis, elevated brain Phe competitively inhibits the activities of tyrosine hydroxylase (TH) and tryptophan hydroxylase 2 (TPH2), the enzymes catalyzing the rate-limiting steps in neuronal dopamine and serotonin synthesis respectively (Ogawa and Ichinose 2006). Here, in order to more fully understand the pathophysiology of the PKU phenotype, we examined the behavioral and cognitive phenotypes of hyperphenylalaninemic C57BL/6-*Pah*^{enu2/enu2} mice in comparison to their wild type and *Pah*^{enu2} heterozygous littermates. In addition, we report the effects of two different Phe-reducing treatments, namely dietary Phe restriction or liver-directed gene therapy with a PAH-expressing recombinant adeno-associated virus serotype 8 (rAAV2/8) vector (**Figure 1**), upon blood and brain amino acid and brain monoamine neurotransmitter contents and upon the behavioral and cognitive phenotypes of *Pah*^{enu2/enu2} mice. Our hypotheses were that correction of serum and consequently brain phenylalanine concentrations following administration of either dietary phenylalanine restriction or liver gene therapy to restore liver PAH activity would lead to functionally increased brain TH and TPH2 activity and correction of brain dopamine and serotonin content in hyperphenylalaninemic *Pah*^{enu2/enu2} mice. Furthermore, we proposed that correction of brain monoamine neurotransmitter content would be associated with improvement in the behavioral and cognitive phenotypes of hyperphenylalaninemic *Pah*^{enu2/enu2} mice.

Figure 1. Phenylketonuria, effects of hyperphenylalaninemia upon brain monoamine neurotransmitter metabolism, and phenylalanine-lowering treatments applied in this work.



Pah^{enu2/enu2} mice lack phenylalanine hydroxylase (PAH) activity in liver and cannot convert L-phenylalanine (Phe) to L-tyrosine (Tyr). Tyr and L-tryptophan (Trp) are the substrates for dopamine (DA) and serotonin synthesis respectively in brain. Both amino acids cross the blood brain barrier and enter neurons via diffusion facilitated by the LAT1 neutral amino acid transporter in competition with Phe and other large neutral amino acids. Tyr is converted to L-DOPA by tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis. TH activity is stimulated by Tyr, but inhibited through Phe-mediated competition, DA or L-DOPA-mediated feedback inhibition, and via a DA-activated, G-protein coupled, synthesis-modulating autoreceptor. TH activity is also regulated through reversible phosphorylation triggered by a variety of stimuli (not shown). DA is synthesized from L-DOPA by aromatic amino acid decarboxylase (*AADC*), taken up into secretory vesicles via a monoamine specific transporter (*VMAT2*) and secreted into the synapse following Ca^{+2} influx in response to a nerve stimulus. Neurotransmission terminates through DA reuptake via a specific transporter (*DAT*) or through degradation of DA to homovanillic acid (HVA), hence brain HVA content can be taken as a measure of DA turnover. Extracellular DA can also act via another G protein-coupled autoreceptor to suppress further synaptic DA release. Analogous mechanisms regulate the synthesis and secretion of serotonin from Trp followed by degradation to 5-hydroxyindoleacetic acid (5-HIAA). The present work has been based upon the hypothesis that correction of serum and consequently brain phenylalanine concentrations following administration of either DIETARY PHENYLALANINE RESTRICTION or AAV8-mediated LIVER GENE THERAPY to restore liver PAH activity would lead to functionally increased brain TH and TPH2 activity and correction of brain dopamine and serotonin content in hyperphenylalaninemic *Pah*^{enu2/enu2} mice. Furthermore, we proposed that correction of brain monoamine neurotransmitter content would be associated with improvement in the behavioral and cognitive phenotypes of hyperphenylalaninemic *Pah*^{enu2/enu2} mice.

2. MATERIALS AND METHODS

2.1 Animal husbandry. Animal care and experimentation were performed in accordance with the guidelines of the Dept. of Comparative Medicine, Oregon Health & Science University and the National Institutes of Health Guide for the Care and Use of Laboratory animals. C57Bl/6-*Pah*^{enu2/enu2} mice (*Pah*^{-/-}), derived from the original BTBR-*Pah*^{enu2/enu2} strain (McDonald et al. 1990), are homozygous for a missense mutation in exon 7 of the murine *Pah* gene, are completely deficient in liver PAH activity, are consequently hyperphenylalaninemic on an unrestricted diet, and are a representative animal model of human PKU. Genotyping for the presence of the *Pah*^{enu2} mutation was performed by a PCR-based analysis (Harding et al. 1998) or a Taqman quantitative PCR assay. Control animals including wild type C57Bl/6 mice (*Pah*^{+/+}) and *Pah*^{enu2/+} heterozygotes, which were co-reared littermates of *Pah*^{-/-} mice resulting from *Pah*^{enu2/+} X *Pah*^{enu2/+} breedings in our mouse colony. For the first month after weaning until the onset of the experimental trial at two months age, all mice were fed tap water and standard mouse chow (Lab Diet Picolab Rodent diet 20, PMI Nutrition International, St. Louis, MO) *ad libitum* providing approximately 24% protein and 1.04% L-phenylalanine by weight. Given that adult mice consume approximately 5 g chow per day, daily L-phenylalanine intake is estimated to be approximately 50 mg per day. The animals were housed under a standard 12 hours on, 12 hours off light cycle. All surgical procedures were carried out with inhaled isoflurane general anesthesia to minimize pain and discomfort.

2.2 Dietary phenylalanine restriction trial. Eighteen *Pah*^{-/-} mice (9 males and 9 females) were switched from standard mouse chow to a phenylalanine (Phe)-restricted diet at 8 weeks age. The animals consumed a Phe free mouse chow (Harlan Teklad TD.01642); their drinking water was supplemented with L-phenylalanine (Sigma-Aldrich), 50 mg/100 ml (0.05%) to prevent Phe deficiency. Given a typical drinking water intake of 3 ml per day, the estimated daily Phe intake on this regimen was 0.15 mg per day, and this amount of Phe supplementation was known from previous work to maintain blood phenylalanine in *Pah*^{-/-} mice to within or slightly above the normal range of blood phenylalanine in wild type mice. This diet was continued for eight weeks and then the animals underwent detailed behavioral and cognitive testing as described below. The low Phe diet was continued through the testing period until euthanasia at 18-20 weeks age for a total exposure of 10-12 weeks dietary Phe restriction. As controls, fourteen

additional Pah^{-/-} mice (6 males and 8 female) and eighteen Pah^{+/+} or Pah^{+/-} mice, littermates to the low phenylalanine diet group, continued to receive standard mouse chow (23.9% protein and 1.05% Phe by weight) and drinking water through the same period and were also assessed using the same behavioral and cognition testing protocol.

2.3 Liver-directed adeno-associated virus vector-mediated gene therapy. Eleven Pah^{-/-} mice (4 males and 7 females) were treated with PAH-expressing recombinant adeno-associated virus serotype 8 (rAAV2/8 vector) by portal vein injection at 8 weeks age. This rAAV2/8 vector harbors the murine Pah cDNA under the transcriptional control of a strong liver specific promoter (LSP) and has been previously shown by our laboratory to correct blood Phe in treated Pah^{-/-} mice (Harding et al. 2006). The rAAV2/8 vector was prepared by the OHSU Viral Vector Core using triple transfection of the required recombinant plasmids into cultured 293K cells and purification by cesium chloride density centrifugation using standard methods. The titer of viral genomes in the preparation was determined by quantitative PCR. Each animal received 2.5×10^{11} rAAV2/8 vector genomes (vg) in 100 μ l sterile saline by open laparotomy and direct injection into the portal vein. Treated mice continued to receive standard mouse chow and drinking water *ad libitum* for eight weeks following rAAV2/8 injection before undergoing behavioral and cognitive assessment at 16 weeks age. The mice were euthanized at 18-20 weeks age for tissue harvest. Ten Pah^{+/+} and eleven additional Pah^{-/-} mice, littermates of the rAAV2/8 treated group, did not receive any AAV treatment, continued on standard mouse chow and drinking water, and served as contemporaneous controls for behavioral, cognitive, and biochemical analyses.

2.4 Behavioral and cognitive testing. All mice were tested in the following behavioral and cognitive tests in the following order: open field, rotorod, and nest building (week 1), water maze (week 2), passive avoidance and contextual and cued fear conditioning (week 3). The open field and water maze tests are described below. The rotorod, contextual and cued fear conditioning were performed as previously reported (Olsen et al. 2013). The passive avoidance test was performed as described (Benice and Raber 2009). The referenced methods are not further described here because we found no effects of either HPA or treatment upon these measures.

2.5 Open field: Mice were placed for ten minutes in a 40.64 cm X 40.64 cm brightly lit open arena. The movement and location of the mice was recorded with Noldus Ethovision video tracking (Wageningen, The Netherlands) and was used to assess exploratory behavior and measures of anxiety. The center zone (20.32 x 20.34 cm) and peripheral zone (remaining part of the open field) were also analyzed separately to assess measurements of anxiety. More anxious mice in the open-field spend less time in the more anxiety-provoking center area (Choleris et al. 2001).

2.6 Nest building: Nest building was analyzed as described (Johnson et al. 2015). Briefly, animals were singly housed and provided with two cotton squares to build nests. 48 hours after being housed, photos were taken of the nests built. Two researchers blinded to treatment then rated the complexity of the nest on a scale from 1 to 5, where 1 indicates that the mouse has not torn the cotton squares apart at all, and 5 indicates a finished, highly complex nest. The scores from the two researchers were averaged for analysis.

2.7 Water maze: Ability to navigate to a visible platform was evaluated using the water maze and Ethovision video tracking software. The maze (circular tub, 122 cm diameter) contained a platform marked by a beacon. The location of the platform was changed between sessions. There were a total of five sessions. Each session consisted of two trials. Each test day consisted of 2 sessions, one in the morning and one in the afternoon. Each trial was up to 60 seconds with a 10-15 minute inter-trial interval. If the mouse did not find the platform in 60 seconds then it was led to the platform by the experimenter and allowed to remain there for 3 seconds before being removed from the maze. Mice were placed in the water maze facing the wall of the pool at nine different drop locations around the pool. The drop location was alternated for each trial. Time to reach the platform (latency), cumulative distance to the platform, and swim speeds were analyzed. As the PAH-/- mice showed extremely poor ability to locate the visible platform, no training sessions with the platform hidden were administered following the visible platform training sessions.

2.8 Euthanasia and tissue harvest. Animals were sedated using inhaled isoflurane anesthesia. Whole blood was collected by cardiac puncture, allowed to clot in an Eppendorf tube, and serum was separated by centrifugation. The mice were then

euthanized by exsanguination and perfused with 20 ml normal saline via the left cardiac ventricle to clear blood from the cerebral circulation. Following decapitation, whole brain was rapidly excised from the cranium, split sagittally, and immediately submerged in liquid nitrogen. Half brains and serum samples were stored at -80°C until processing for amino acid or neurotransmitter analysis.

2.9 Amino acid analysis. Amino acid concentrations in sera or brain tissue were measured by pre-column derivitization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC, Waters AccQ Tag™ derivitization system) and separation by ultra high performance liquid chromatography and UV absorbance detection (Waters Acquity™ UPLC, Milford, MA) using the Waters Masstrak Amino Acid Analysis method. Serum samples were de-proteinized by adding an equal volume of 10% sulfosalicylic acid containing the non-physiological amino acid norvaline (250 µM) as an internal recovery standard. For analysis of amino acid concentrations in brain, half-brains were mechanically homogenized in 5 volumes/weight 10% trichloroacetic acid containing norvaline (150 µM) and processed as previously described (Winn et al. 2016). Serum amino acid concentrations were reported as µM and corrected for dilution to reflect the actual concentrations in serum. The measured amino acid concentrations in the brain homogenates were corrected for the tissue wet weight and reported as nmol/gm wet brain weight.

2.10 Brain monoamine neurotransmitter analysis. Mouse half-brains were mechanically homogenized in ice-cold homogenizing buffer (50 mM Tris-HCl, pH 7.5, 0.1 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 µM leupeptin, and 1 µM pepstatin), 4 µl/mg tissue and further processed according to a previously published method (Elzaouk et al. 2003). Monoamine neurotransmitter concentrations (L-DOPA, dopamine, HVA, 5-HTP, serotonin, and 5-HIAA) were measured in brain tissue by HPLC and electrochemical detection (Blau et al. 1999). Measured brain homogenate monoamine neurotransmitter concentrations were corrected for the protein content of the homogenate and expressed as pmol/mg protein.

2.11 Measurement of brain TH and TPH abundance and activities. Immunodetection of TH and TPH protein relative to GAPDH protein and measurement of maximally

stimulated TH and TPH activities in mouse half-brain homogenates were carried out essentially as previously described (Calvo et al. 2010).

2.12 Brain TH and TPH immunohistology. Select animals from each experimental cohort underwent saline-paraformaldehyde perfusion in preparation for brain fixation and immunohistology. Under deep isoflurane inhaled anesthesia, mice were perfused with 0.9% saline via cardiac infusion to clear the cerebral circulation of blood and then were perfused with an equal volume of fixative solution (4% paraformaldehyde in phosphate buffered saline). Excised whole brains were then fixed for 24 hours in the same fixation solution followed by dehydration in 25% buffered sucrose for an additional 24 hours. 15 micrometer coronal frozen sections were then cut from the regions of the striatum using a cryostat and mounted on glass slides. Sections were treated (following blocking) with rabbit polyclonal anti-TH antibody (1:200 dilution, EMD Millipore Corp. AB152) and incubated for 2 hours at room temperature. After appropriate washes, sections were treated with Alexa 488-conjugated goat anti-rabbit IgG secondary antibody (1:150 dilution, Thermo Scientific, A27034) and incubated for one hour at room temperature. After washing, cover slips were mounted with Vectashield mounting medium containing DAPI. Images were captured on a Nikon H550L fluorescence microscope.

2.13 Statistical analysis. Biochemical, behavioral, and cognitive, data were analyzed using two-way analyses of variance (ANOVA) with either genotype and sex or sex and treatment as between-subjects factors. The water maze learning curves were analyzed using repeated-measures ANOVA with genotype or treatment as between group variables. Inter-group comparisons were performed post analysis using Tukey's multiple comparison tests when appropriate. We considered $p < 0.05$ as statistically significant. Statistical analyses of biochemical data were performed using GraphPad Prism™ (version 7, San Diego, CA) software. Statistical analyses of behavioral and cognitive data were performed using SPSS software (vs22, Chicago, IL).

3. RESULTS

3.1 Murine HPA is associated with severe CNS serotonin deficiency but minimal disturbance of brain dopamine or large neutral amino acid (LNAA) content.

Five month old adult PAH-deficient C57Bl/6-*Pah*^{enu2/enu2} (*Pah*^{-/-}) mice consuming a standard mouse chow (23.9% protein and 1.05% Phe by weight) exhibit severe HPA and elevated brain Phe in comparison to either *Pah*^{+/+} or *Pah*^{+/-} littermates (**Figure 2** and Supplementary Table 1). The mean serum Phe concentration (**Figure 2A**) in male *Pah*^{-/-} mice (2198 ± 71 μ M) was increased more than ten-fold over male *Pah*^{+/+} (132 ± 16 μ M) or *Pah*^{+/-} mice (98 ± 23 μ M). Female *Pah*^{-/-} mice exhibited even greater blood Phe concentration (2654 ± 87 μ M) in comparison to female *Pah*^{+/+} (111 ± 11 μ M) or *Pah*^{+/-} mice (72 ± 7 μ M). Two-way ANOVA revealed significant effects of both genotype ($F(2,47) = 716$, $p < 0.0001$) and sex ($F(1,47) = 4.24$, $p < 0.0451$) upon serum phenylalanine (Phe). This sex difference in serum Phe of *Pah*^{enu2/enu2} mice has been noted previously (Hamman et al. 2005; Ding et al. 2008), but to the best of our knowledge has not been described in humans with PKU. Brain Phe concentration (**Figure 2B**) was similarly increased in *Pah*^{-/-} mice (male *Pah*^{-/-} mice = 713 ± 18 nmol/gm wet brain weight, female *Pah*^{-/-} mice = 811 ± 17 nmol/gm) in comparison to either *Pah*^{+/+} (male = 110 ± 28 nmol/gm, female = 85 ± 15 nmol/gm) or *Pah*^{+/-} mice (male mice = 194 ± 13 nmol/gm, female = 171 ± 14 nmol/gm). A plot of brain phenylalanine content vs. serum phenylalanine concentration in all mice (including untreated mice of all three genotypes and all *Pah*^{-/-} mice treated with dietary Phe restriction or liver-directed gene therapy described below) demonstrated a tight correlation between Phe concentration in blood and brain ($r^2 = 0.914$, $p < 0.0001$, **Figure 2C**).

The LNAA (including Phe, tyrosine, tryptophan, leucine, isoleucine, valine, methionine, threonine, and histidine) move from the circulation across the blood brain barrier through sodium-independent diffusion (Choi and Pardridge 1986) facilitated by the LNAA transporter LAT1 (also designated SCLA7A5), a member of the APC family of transmembrane transport proteins (Kanai et al. 1998; Mastroberardino et al. 1998). LAT1 is expressed on both the luminal (blood side) and the abluminal (extra cellular fluid (ECF) side) of the brain capillary endothelial cell (Sanchez del Pino et al. 1995) and mediates movement of LNAA down a concentration gradient from blood through the endothelial cell and to the ECF compartment. The affinity of the LAT1 transporter for

LNAAs are high relative to blood concentrations of the amino acids ($K_m \sim 20\text{--}200 \mu\text{M}$) suggesting that at normal physiologic plasma LNAAs concentrations the LAT1 transporter is already saturated. However, in this study brain Phe content never appeared to saturate despite massively increased blood Phe suggesting that, during the hyperphenylalaninemic state at least, Phe uptake into brain is mediated through some other mechanism beyond LAT1-mediated facilitated diffusion.

Tyrosine (Tyr) is the product of the PAH reaction and the substrate for dopamine synthesis. In PAH-deficient mice and humans, Tyr becomes a dietary essential amino acid as it is no longer synthesized from Phe. As expected serum Tyr concentration (**Figure 2D**) was significantly decreased in both male and female *Pah*^{-/-} mice ($36 \pm 3 \mu\text{M}$ and $44 \pm 3 \mu\text{M}$ respectively) in comparison to *Pah*^{+/+} (male = $72 \pm 4 \mu\text{M}$, female = $91 \pm 4 \mu\text{M}$) or *Pah*^{+/-} (male = $57.5 \pm 3.6 \mu\text{M}$, female = $56.6 \pm 5.4 \mu\text{M}$) mice. However, despite the greater than 50% decrease in blood Tyr and the massively increased blood Phe of hyperphenylalaninemic mice, brain Tyr content (**Figure 2E**) was not significantly decreased in these animals. Brain Tyr in *Pah*^{-/-} mice (male = $79 \pm 18 \text{ nmol/gm}$, female = $91 \pm 13 \text{ nmol/gm}$) was not significantly different from that of *Pah*^{+/+} (male = $101 \pm 22 \text{ nmol/gm}$, female = $88 \pm 11 \text{ nmol/gm}$) or *Pah*^{+/-} (male = $173 \pm 18 \text{ nmol/gm}$, female = $163 \pm 20 \text{ nmol/gm}$) animals. Furthermore, analyses of brain Tyr vs. serum Tyr ($r^2 = 0.013$) or of brain Tyr vs. serum Phe ($r^2 = 0.059$) failed to demonstrate any significant correlations (not shown).

In dopaminergic neurons, Tyr is converted to dopamine with the rate of conversion limited by the activity of tyrosine hydroxylase (TH) enzyme (**Figure 1**). Following synaptic release and signal transmission, dopamine may either be taken back up into the axon of the dopaminergic neuron via a dopamine-specific transporter (DAT) or enzymatically converted to homovanillic acid (HVA). Impairment of systemic Tyr synthesis in PKU has led to the hypothesis that CNS dopamine deficiency contributes to the neurobehavioral phenotypes associated with PKU. However, in our study, HPA and serum Tyr deficiency were associated with only minimal impairment of dopamine synthesis and turnover. Two-way ANOVA revealed an effect of genotype ($p < 0.0001$) upon brain dopamine content, but post hoc group comparisons of the normalized data demonstrated a significant difference in brain dopamine only between female *Pah*^{+/+} and female *Pah*^{-/-} mice ($p < 0.01$) (**Figure 2F**). In that comparison, female *Pah*^{+/+} brain

dopamine content was on average 55% higher than that of female Pah^{-/-} mice. For males, a trend for 21% higher brain dopamine in Pah^{+/+} mice than in Pah^{-/-} animals was measured. The brain contents of HVA (**Figure 2G**) of female Pah^{+/+} and Pah^{+/-} mice were on average 65% and 69% greater ($p < 0.0001$) respectively than that of Pah^{-/-} mice, whereas for males, only a trend (26% and 14% higher for Pah^{+/+} and Pah^{+/-} mice, respectively) was measured. Recalling that brain Tyr content was not significantly different among the different genotypes and that brain Phe concentrations were higher in female than in male Pah^{-/-} mice, these data suggest that Phe-mediated competitive inhibition of TH activity rather than substrate availability is likely responsible for the minimal deficiency in brain dopamine content and turnover measured in hyperphenylalaninemic (and particularly in female) Pah^{-/-} mice.

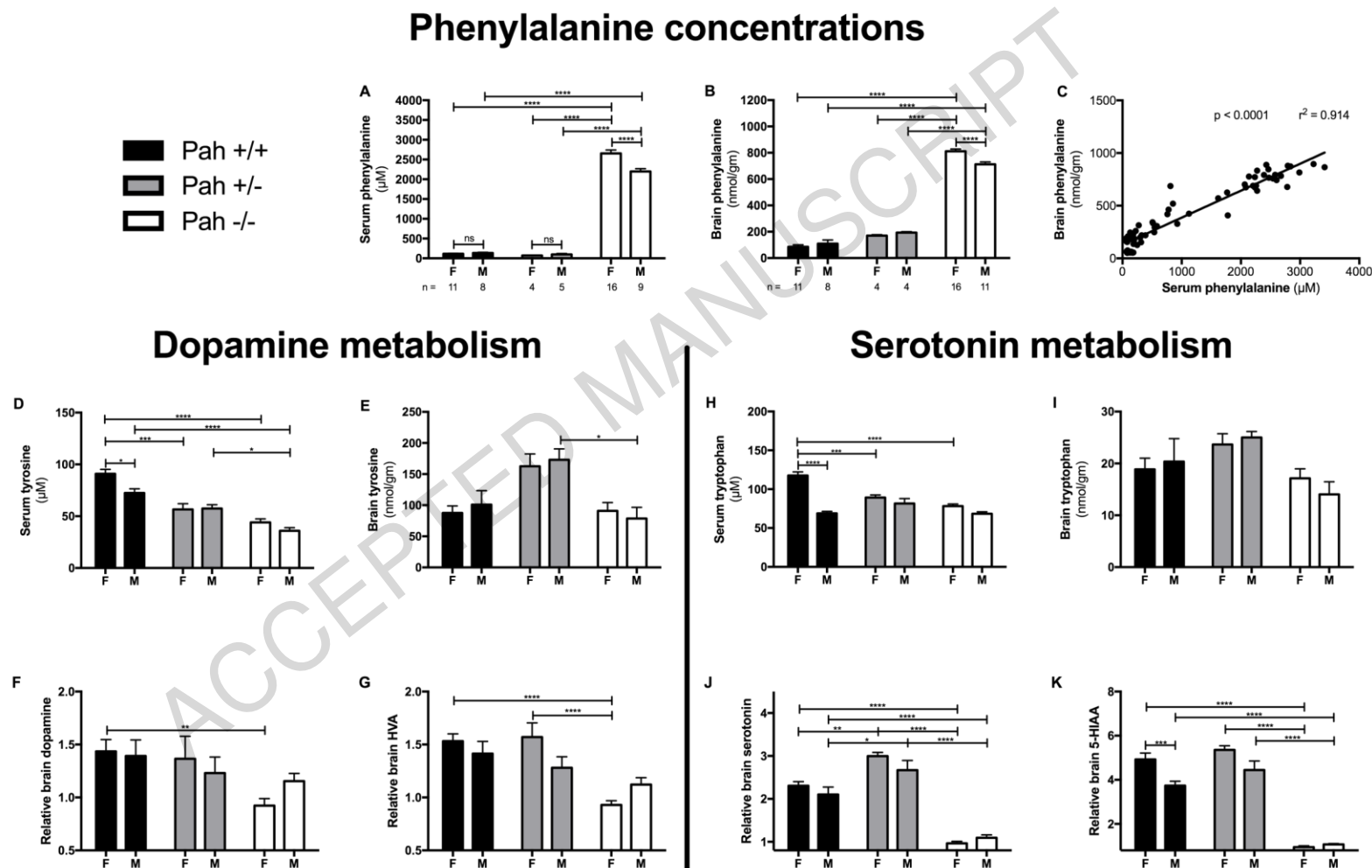
Serotonin is synthesized in the serotonergic neurons from L-tryptophan (Trp) via the rate-limiting enzyme, TPH isoform 2 (TPH2). We measured a less than twofold increase in serum Trp concentration of female Pah^{+/+} mice ($118 \pm 4.6 \mu\text{M}$) in comparison to male Pah^{+/+} mice ($69 \pm 2.5 \mu\text{M}$) or to Pah^{+/-} ($89 \pm 3.4 \mu\text{M}$) or Pah^{-/-} ($78 \pm 2.6 \mu\text{M}$) female mice (**Figure 2H**). These differences are unexplained as no such differences were seen in comparing male Pah^{+/+} ($69 \pm 2.5 \mu\text{M}$), Pah^{+/-} ($82 \pm 6.3 \mu\text{M}$) or Pah^{-/-} ($68 \pm 2.5 \mu\text{M}$) mice. Brain Trp content was not significantly different between the genotypes or sexes (**Figure 2I**). As was true for Tyr, there was no clear correlation between brain Trp and serum Trp or brain Trp and serum Phe (data not shown).

The brain content of serotonin (**Figure 2J**) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) (**Figure 2K**) was severely decreased in hyperphenylalaninemic mice, of both sexes, in comparison to either Pah^{+/+} or Pah^{+/-} mice. In female mice, brain serotonin was on average 240% greater in Pah^{+/+} and 310% greater in Pah^{+/-} than in Pah^{-/-} mice. Similarly, male Pah^{+/+} mice exhibited 192% and male Pah^{+/-} mice 244% greater brain serotonin content than male Pah^{-/-} mice. Similar results were found for brain 5-HIAA content. Mean brain 5-HIAA contents of female Pah^{+/+} (519%) and female Pah^{+/-} (564%) were dramatically higher than in female Pah^{-/-} mice. Likewise, male Pah^{+/+} (347%) and Pah^{+/-} (413%) brain 5-HIAA contents were significantly greater than that of Pah^{-/-} mice.

The severe deficiency of the brain serotonin pathway in *Pah*^{-/-} mice is associated with extreme HPA and elevated brain Phe content but normal brain Trp content. Our interpretation of these data, similar to our conclusions from the dopamine pathway data, is that Phe-mediated competitive inhibition of brain TPH2 activity rather than deficiency of the substrate L-tryptophan is primarily responsible for serotonin deficiency in murine PKU.

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Figure 2. Effects of genotype upon amino acids and monoamine neurotransmitters in C57BL/6-*Pah*^{enu2/enu2} mice.



Serum and brain amino acids and brain monoamine neurotransmitter content in female (**F**) and male (**M**) Pah^{+/+}, Pah^{+/-}, Pah^{-/-} mice. Data expressed as mean \pm SEM. The data were analyzed using two-way ANOVA with post hoc intergroup comparisons by Tukey's multiple comparison test. Horizontal bars and asterisks above the columns indicate the probability (p) from the Tukey's test that the means of the selected groups are identical. **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$. Serum amino acid concentrations are expressed as μM , brain amino acid content as nmol/gm brain wet weight, and brain monoamine neurotransmitters and their metabolites are expressed relative to the mean of untreated Pah^{-/-} mice (both genders combined). (**A**) Serum phenylalanine, (**B**) brain phenylalanine. (**C**) Plot of brain phenylalanine vs. serum phenylalanine combining data from all groups of mice (including Pah^{-/-} mice undergoing Phe-reducing treatment). Linear regression analysis reveals a probability of $p < 0.0001$ that the slope of the curve is equal to zero and suggests a strong correlation between the serum and brain phenylalanine concentrations. (**D**) Serum tyrosine, (**E**) brain tyrosine, (**F**) relative brain dopamine, (**G**) relative brain homovanillic acid (HVA), (**H**) serum tryptophan, (**I**) brain tryptophan, (**J**) relative brain serotonin, (**K**) relative brain 5-hydroxyindoleacetic acid (5-HIAA).

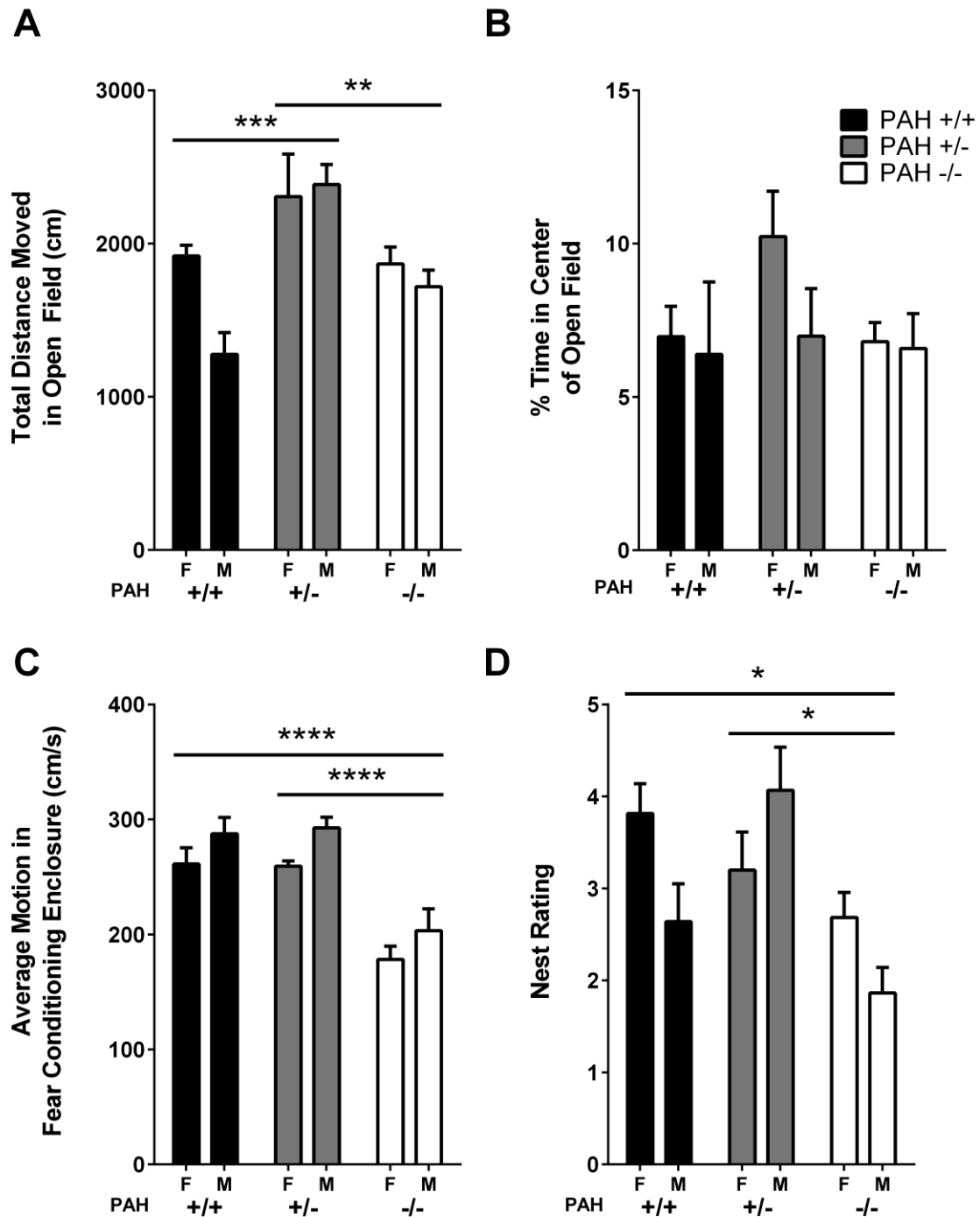
3.2 Lifelong exposure to HPA in untreated adult *Pah^{enu2/enu2}* mice is associated with altered exploratory activity in the open field and in the fear conditioning enclosure, and deficits in nesting behavior and task learning in the water maze.

Prior to euthanasia for the above biochemical evaluations, detailed behavioral and cognitive assessments of all experimental mice were carried out. These assessments included nest building behavior and performance in the open field, on the rotorod, in the water maze, in a passive avoidance test, and in the contextual and cued fear conditioning tests. Comparison of these results in *Pah*^{-/-} mice to those of *Pah*^{+/+} and *Pah*^{+/-} mice revealed significant behavioral effects of chronic HPA only upon exploratory activity measured in the open field (**Figure 3A**), baseline average motion in the fear conditioning enclosure (**Figure 3C**), and upon nesting behavior (**Figure 3D**). Additional effects of chronic HPA were seen in task learning in the Morris water maze (**Figures 4B, 4C, and 4D**). In the open field, two-way ANOVA revealed an effect of genotype ($p < 0.0001$) and a trend toward an effect of sex ($p = 0.0509$) upon the distance moved. Post hoc Tukey's multiple comparisons revealed that *Pah*^{+/-} mice moved more than *Pah*^{+/+} ($p < 0.001$) and *Pah*^{-/-} ($p < 0.01$) mice. There were no effects of either genotype or sex upon the % time spent in the center of the open field, a measure of open field anxiety (**Figure 3B**). In the fear conditioning enclosure, there was also a genotype effect ($p < 0.0001$). *Pah*^{-/-} mice moved less than *Pah*^{+/+} ($p < 0.0001$) and *Pah*^{+/-} mice ($p < 0.0001$) (**Figure 3C**). Thus, as in the open field, *Pah*^{-/-} mice moved less than *Pah*^{+/-} mice, but unlike in the open field, there were no differences in activity between the *Pah*^{+/+} and *PAH*^{+/-} mice. This difference might be due to the differences between an open and a closed environment and in the different floor types involved in these two distinct tests. There was an effect of genotype on nest building ($p = 0.0014$) (**Figure 3D**). *PAH*^{-/-} mice built less elaborate nests than *PAH*^{+/-} mice ($p < 0.01$) or *PAH*^{+/+} mice ($p < 0.05$). Negative results from the other assessments are not specifically reported in detail here.

Water maze testing of untreated *Pah*^{-/-} mice demonstrated severe cognitive deficits in the animals (**Figures 4B, 4C, and 4D**) with a significantly increased total distance moved and cumulative to and time to reach (latency) the visible platform for *Pah*^{-/-} animals. First, average swim speeds were analyzed to determine if differences in swim speeds accounted for the differences in task performance (**Figure 4A**). A two-way ANOVA did not show any significant differences between the genotypes ($p = 0.149$) or

sexes ($p = 0.585$), suggesting that the performance differences seen were not due to group differences in swim speeds. Two-way ANOVA revealed effects on cumulative distance to the platform of both genotype ($p < 0.0001$) and sex ($p = 0.021$), with an interaction between genotype and sex ($p = 0.024$). Post hoc comparison revealed significant differences between all genotypes (Pah+/+ vs. Pah+/- - $p = 0.014$, Pah+/+ vs. Pah-/- - $p < 0.0001$, and Pah+/- vs. Pah-/- - $p < 0.0001$). Similar results were obtained for latency with effects of genotype ($p < 0.0001$), sex ($p = 0.008$), and an interaction between genotype and sex ($p = 0.013$), and similar differences between all genotypes in the post hoc comparisons. Females showed a higher cumulative distance to the visible platform, indicative of reduced cognitive performance, compared to males. Analysis of total distance moved again showed similar results, with effects of genotype ($p < 0.001$) and sex ($p = 0.015$). Overall, the results document severe cognitive impairments in chronically hyperphenylalaninemic Pah-/- mice.

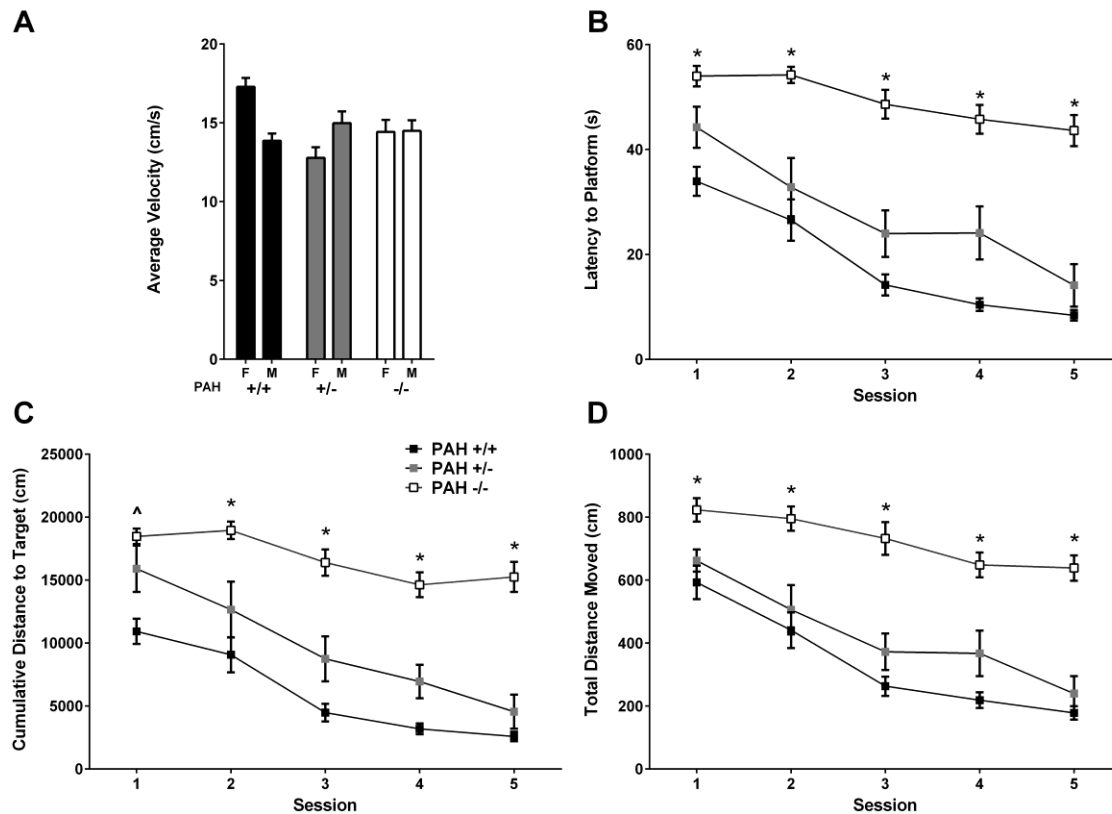
Figure 3. Behavioral performance of C57BL/6-*Pah*^{enu2/enu2} mice.



Results of behavioral (open field, fear conditioning, and nesting behavior) assessments in female (F) and male (M) Pah+/, Pah+/-, and Pah-/- mice. The results of the behavioral performance in the open field are presented in panel (A) open field activity (total distance moved) and (B) open field anxiety (% time in center). Baseline activity

while in the fear conditioning enclosures is shown in panel (C). Nest building (qualitative nest rating scale) is presented in panel (D). All data are mean \pm SEM. Pah+/- mice showed higher activity levels than Pah+/+ mice and Pah-/- mice in the open field. Pah+/+ and Pah+/- mice both showed higher baseline activity than Pah-/- mice in the fear conditioning enclosure. Pah-/- mice build less elaborate nests than Pah+/- mice and Pah+/+ mice. In Pah-/- mice, females built more elaborate nests than males. Pah+/+ and Pah+/- mice showed better nesting behavior than Pah-/- mice ($p < 0.05$, $p < 0.01$, respectively). Vertical bars and asterisks above the columns indicate the probability (p) from the Tukey's test that the means of the selected groups are identical. **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$.

Figure 4. Cognitive performance of C57BL/6-*Pah*^{enu2/enu2} mice.



Performance in the water maze is presented with both sexes combined. The results are shown in panel (A) average velocity (cm/second), (B) latency to the target platform (seconds) (C) cumulative distance to the target (cm) and (D) total distance moved (cm). The data are represented as mean \pm SEM for each experimental group at each individual session. (A). There were no differences in swim speeds over the five sessions ($p > 0.05$). (B). Latency to locate the target platform in the water maze. *Pah*^{+/+} mice showed better performance than *Pah*^{+/-} ($p = 0.020$) and *Pah*^{-/-} mice ($p = 0.001$) and located the platform in less time. In addition, *Pah*^{+/-} mice showed better performance than *Pah*^{-/-} mice ($p < 0.001$). (C) Similar results were seen when cumulative distance to the target location was analyzed. *Pah*^{+/-} mice showed better performance than *Pah*^{-/-} mice ($p < 0.001$) and swam on average closer to the platform location. There was a genotype \times session interaction ($F(8,224) = 3.057$, $p = 0.003$). There was an effect of genotype in all Sessions (Session 1: *Pah*^{+/+} vs *Pah*^{-/-}, $p < 0.001$; *Pah*^{+/+} vs *Pah*^{+/-} $p = 0.009$; Session 2: *Pah*^{+/+} vs *Pah*^{-/-}, $p < 0.001$; *Pah*^{+/+} vs *Pah*^{+/-}, $p = 0.003$; Session 3: *Pah*^{+/+} vs *Pah*^{-/-}, $p = 0.000$; *Pah*^{+/+} vs *Pah*^{+/-}, $p < 0.001$; Session 4: *Pah*^{+/+} vs *Pah*^{-/-}, p

< 0.001; Pah+/- vs Pah-/-, $p < 0.001$; Session 5: Pah+/+ vs Pah-/-, $p < 0.001$; Pah+/- vs Pah-/-, $p < 0.001$). In the Pah-/- mice, females showed a higher cumulative distance to the platform than males. (D) Analysis of the total distance moved confirmed the results of the other outcome measures. Both Pah+/+ and Pah+/- mice showed better performance than Pah-/- mice ($p < 0.001$). The asterisks denote sessions in which performance of the experimental group (Pah+/- or Pah-/-) differed significantly from those of Pah+/+ mice.

3.3 Reduction of blood and brain Phe through either dietary phenylalanine restriction or liver-directed gene therapy ameliorates brain monoamine neurotransmitter deficiency in murine PKU.

We evaluated the effectiveness of two different blood Phe reducing treatment methods, either dietary Phe restriction or liver-directed gene therapy with a PAH expressing recombinant adeno-associated virus pseudotype 8 (rAAV2/8) vector (Harding et al. 2006) for their ability to correct amino acid abnormalities and brain monoamine neurotransmitter deficiencies in Pah-/- mice. Gene therapy treated mice received a single portal vein injection of rAA2/8 vector (2.5×10^{11} vector genomes (vg) per mouse) at 8 weeks age and no additional therapy until euthanasia at 18-20 weeks age, while diet treated mice were maintained on a Phe-restricted diet from 8 weeks age until euthanasia at 18-20 weeks. The effects of therapeutic intervention upon the serum and brain aromatic amino acids and brain monoamine neurotransmitters of Pah-/- mice are depicted in **Figure 5** and reported in Supplementary Table 2. Both treatment approaches significantly reduced serum (**Figure 5A**) and brain Phe (**Figure 5B**) in both sexes. In the dietary treatment group, serum Phe concentration was reduced from $2198 \pm 71 \mu\text{M}$ to $295 \pm 67 \mu\text{M}$ in male Pah-/- mice ($p < 0.0001$) and from $2654 \pm 87 \mu\text{M}$ to $494 \pm 111 \mu\text{M}$ in female Pah-/- mice ($p < 0.0001$). Consequently, brain Phe content was also reduced (male Pah-/- mice: untreated – $713 \pm 18 \text{ nmol/gm}$, diet treated – $244 \pm 17 \text{ nmol/gm}$, $p < 0.0001$; female Pah-/- mice: untreated – $811 \pm 17 \text{ nmol/gm}$, diet treated – $326 \pm 41 \text{ nmol/gm}$, $p < 0.0001$). Liver-directed gene therapy at the vector dose used was somewhat less and more variably effective in correcting serum Phe concentrations (rAAV2/8-treated male Pah-/- mice – $407 \pm 175 \mu\text{M}$ with a range of 191-925 μM , rAAV2/8-treated female Pah-/- mice – $961 \pm 220 \mu\text{M}$ with a range from 248-1780 μM) in comparison to dietary therapy but these decreases were still highly significant (p

<0.0001). Brain Phe content was also decreased ($p < 0.0001$) in rAAV2/8-treated mice (rAAV2/8-treated male Pah^{-/-} mice – 206 ± 41 nmol/gm; rAAV2/8-treated female Pah^{-/-} mice – 408 ± 72 nmol/gm). At euthanasia four months after rAAV2/8 injection, the mean number of rAAV2/8 vector genomes (vg) detected in total DNA extracted from a liver biopsy and measured by quantitative PCR was only 2.6 ± 0.6 vg per haploid liver genome (mean \pm SE). Mean liver PAH activity in male rAAV2/8-treated mice was $10.4\% \pm 0.4\%$ (mean \pm SE) of wild type liver PAH activity and only $7.6\% \pm 0.3\%$ in female rAAV2/8-treated mice. Liver PAH activity in untreated Pah^{-/-} mice was below the limit of detection for this radiometric assay (1% wild type liver PAH activity) as we have previously reported (Harding et al. 1998). In our initial work with rAAV2/8 gene therapy (Harding et al. 2006), serum Phe was corrected to wild type levels only in mice that had received a rAAV2/8 vector dose $> 5 \times 10^{11}$ vg/mouse yielding liver rAAV2/8 vg copy number > 25 vg/haploid liver genome and liver PAH activity greater than approximately 10% wild type liver PAH activity. In this experiment, the lower rAAV2/8 dose we employed was associated with only partial restoration of liver PAH activity and serum Phe concentrations that were significantly decreased but still above the normal range. Neither dietary Phe restriction nor liver-directed gene therapy in this experiment led to correction of blood or brain Phe down to concentrations measured in Pah^{+/+} or Pah^{+/-} mice; however, the serum Phe levels achieved with diet therapy are in line with the typical therapeutic goal of reducing serum phenylalanine to below $360 \mu\text{M}$ in humans with PKU (Vockley et al. 2014).

Reduction of blood phenylalanine by either treatment method is associated with reversal of HPA-associated hypopigmentation in the mice (**Figure 5C**). Hypopigmentation in PAH deficiency is caused by Phe-mediated competitive inhibition of the melanin synthesis enzyme tyrosinase (Miyamoto and Fitzpatrick 1957); correction of HPA releases tyrosinase inhibition and restores melanin synthesis.

Despite the variable effect of rAAV2/8 administration upon serum and brain Phe, liver-directed gene therapy yielded complete correction of serum Tyr in treated Pah^{-/-} mice (**Figure 5D**) (rAAV2/8-treated male Pah^{-/-} mice – $98.3 \pm 9.5 \mu\text{M}$ vs. $35.9 \pm 3.0 \mu\text{M}$ in untreated mice ($p < 0.0001$); rAAV2/8-treated female Pah^{-/-} mice – $86.4 \pm 9.8 \mu\text{M}$ vs. $44.1 \pm 3.1 \mu\text{M}$ in untreated female Pah^{-/-} mice ($p < 0.0001$)). Liver-directed gene therapy restores liver PAH activity and therefore restores systemic Tyr synthesis. Despite

leading to more robust correction of HPA in comparison to gene therapy, dietary Phe restriction with the diet formulation used here did not lead to significant improvement in blood Tyr concentration of Pah^{-/-} mice (diet treated male Pah^{-/-} mice – $31.2 \pm 3.4 \mu\text{M}$; diet-treated female Pah^{-/-} mice – $31.7 \pm 2.4 \mu\text{M}$), results that were not statistically different from those of untreated Pah^{-/-} mice. The standard mouse chow utilized in our lab contains 0.71% Tyr (as part of intact dietary protein) but the Phe-free diet we used contained only 0.5% Tyr with the Tyr added to the chow as the free amino acid. The aromatic amino acids including Tyr are relatively insoluble in water in comparison to other amino acids, and therefore free Tyr added to mouse chow may be less available for intestinal absorption in comparison to Tyr released from digested whole protein. For all these reasons, persistently low serum Tyr concentration in Pah^{-/-} mice on the Phe-restricted diet is understandable.

Although serum Tyr increased significantly in rAAV2/8-treated mice, the brain Tyr content was not appreciably altered by liver-directed gene therapy, nor by dietary Phe restriction (**Figure 5E**). Despite that, treatment did still yield a slight but significant effect upon brain dopamine (**Figure 5F**) and HVA (**Figure 5G**) content. In females, brain dopamine content increased 69% on average in diet treated mice in comparison to untreated mice ($p < 0.01$). Dopamine increased 39% on average in rAAV2/8-treated female mice, 14% in rAAV2/8-treated male mice, but was virtually unchanged in diet-treated male mice. None of these latter changes reached statistical significance by post hoc group comparisons however. Brain HVA increased by 60% on average in diet-treated female Pah^{-/-} mice ($p < 0.01$), 37% in rAAV2/8-treated females, 4% in diet treated males, and 8% in rAAV2/8-treated males. Again, none of the differences in these last three groups were found to be statistically significant.

Both dietary Phe restriction and rAAV2/8-mediated gene therapy were associated with slight increases in serum Trp (**Figure 5H**) of Pah^{-/-} mice. Serum Trp concentration increased from $78.3 \pm 2.6 \mu\text{M}$ in untreated female Pah^{-/-} mice to $108 \pm 5.5 \mu\text{M}$ in female Pah^{-/-} ($p < 0.0001$) on the Phe-restricted diet and to $100 \pm 3.2 \mu\text{M}$ in female Pah^{-/-} mice ($p < 0.001$) after gene therapy. Trp is an essential amino acid that cannot be synthesized in the body and must be obtained through dietary intake, so these results suggest that lower blood Phe is associated with either improved intestinal Trp uptake or altered liver release of Trp into blood. However, serum Trp concentrations were only modestly

increased in treated male Pah^{-/-} mice (untreated male Pah^{-/-} mice – $68.4 \pm 2.5 \mu\text{M}$, male Pah^{-/-} mice on Phe-restricted diet – $85.6 \pm 3.2 \mu\text{M}$ ($p < 0.05$), male Pah^{-/-} mice following gene therapy – $86.3 \pm 2.9 \mu\text{M}$ (difference not significant). Regardless of whether this apparent effect of treatment upon serum Trp, primarily in female mice, is real, neither treatment had any significant effect upon brain Trp content (**Figure 5I**).

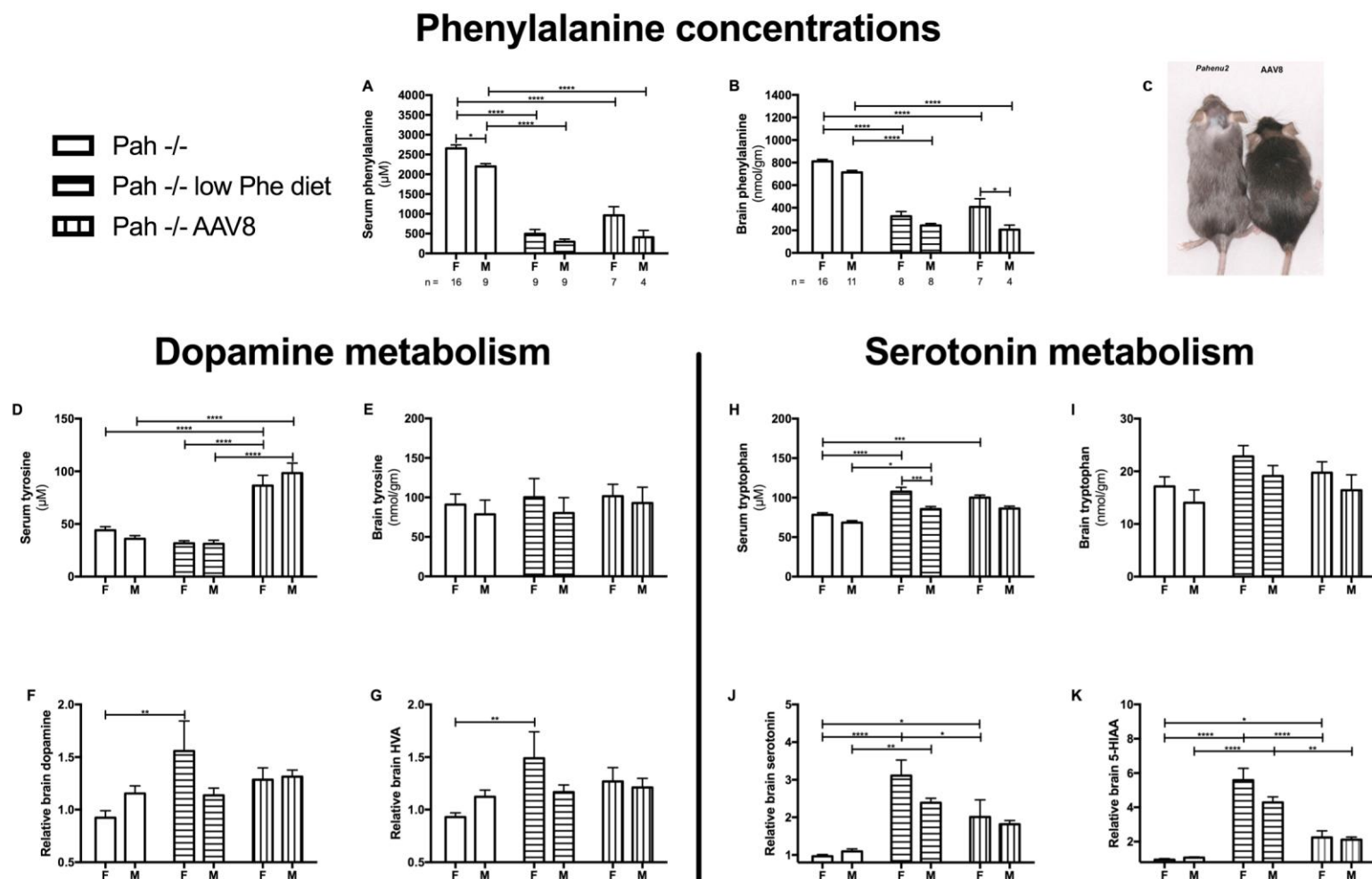
Dietary Phe restriction was associated with complete correction of brain serotonin (**Figure 5J**) and 5-HIAA (**Figure 5K**) content in Pah^{-/-} mice. In female Pah^{-/-} mice, brain serotonin content increased on average by 322% ($p < 0.0001$) and brain 5-HIAA content increased by 588% ($p < 0.0001$) in animals receiving the Phe-restricted diet in comparison to untreated Pah^{-/-} mice. Similar increases were measured in treated male Pah^{-/-} mice (brain serotonin: 218% increase on average in diet-treated mice ($p < 0.01$); brain 5-HIAA: 398% increase ($p < 0.0001$)). Brain serotonin and 5-HIAA content in diet treated female Pah^{-/-} mice (mean brain serotonin = $151 \pm 28 \text{ pmol/mg protein}$; mean brain 5-HIAA = $124 \pm 30 \text{ pmol/mg}$) exceeded that of wild type female Pah^{+/+} animals (brain serotonin = $80 \pm 2.9 \text{ pmol/mg}$; brain 5-HIAA = $73 \pm 5.5 \text{ pmol/mg}$). Similarly, brain serotonin and 5-HIAA in diet-treated male Pah^{-/-} mice (brain serotonin = $110 \pm 7.2 \text{ pmol/mg}$, brain 5-HIAA = $87 \pm 12 \text{ pmol/mg}$) were approximately equal to or greater than levels in Pah^{+/+} mice (brain serotonin = $75 \pm 6.2 \text{ pmol/mg}$, brain 5-HIAA = 51 ± 2.3). These results indicate that reduction of blood and brain Phe in diet treated Pah^{-/-} mice was associated with complete correction of CNS serotonin deficiency.

Brain serotonin and 5-HIAA contents were also increased in Pah^{-/-} mice treated with rAAV2/8-mediated gene therapy (serotonin: female mice – $65.9 \pm 14.9 \text{ nmol/mg}$, male mice – $59.5 \pm 3.3 \text{ nmol/mg}$; 5-HIAA: female mice – $36.2 \pm 6.2 \text{ nmol/mg}$, male mice – $34.1 \pm 2.5 \text{ nmol/mg}$), but the increase was not as robust as seen in the diet-treated group. Average brain serotonin increased 208% ($p < 0.05$) in rAAV2/8-treated female mice but only 166% in male mice, a difference that did not reach statistical significance. Similarly, average brain 5-HIAA increased 237% in rAAV2/8-treated female mice. As discussed above, serum Phe concentration was on average higher in mice treated with rAAV2/8-mediated gene therapy in comparison to animals on the Phe-restricted diet; we propose therefore that the partial correction of blood and brain Phe following treatment with rAAV2/8 vector was responsible for the improved but persistently low brain serotonin and 5-HIAA in comparison to diet-treated mice.

In combining data from all experimental groups, we measured significant negative correlations between brain serotonin and serum Phe, and between brain serotonin and brain Phe (**Supplemental figure 1**).

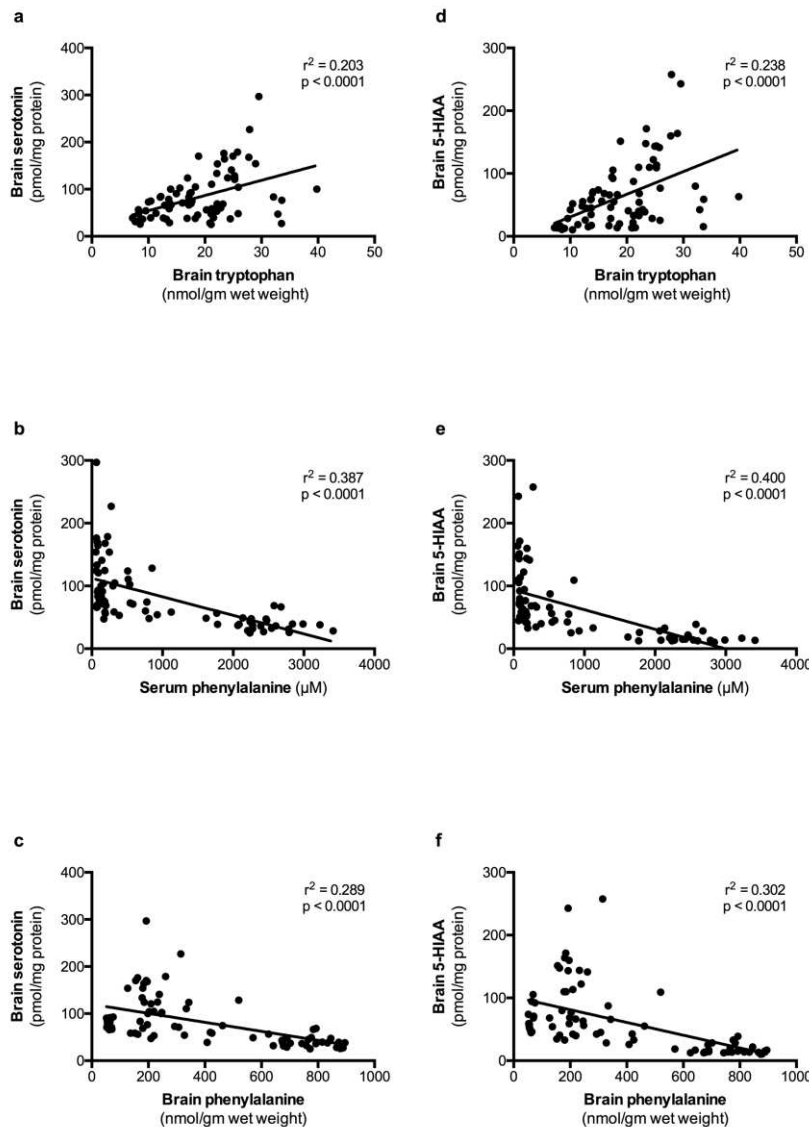
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Figure 5. Effects of phenylalanine-reducing treatments upon amino acids and monoamine neurotransmitters in C57Bl/6-*Pah*^{enu2/enu2} mice.



Serum and brain amino acids and brain monoamine neurotransmitter content in female (**F**) and male (**M**) untreated Pah-/- mice (**Pah-/-**), Pah-/- mice receiving a phenylalanine-restricted diet (**Pah -/- low Phe diet**), and Pah-/- mice following AAV-mediated liver-directed gene therapy (**Pah-/- AAV8**). Data expressed as mean \pm SEM. The data were analyzed using two-way ANOVA with post hoc intergroup comparisons by Tukey's multiple comparison test. Horizontal bars and asterisks above the columns indicate the probability (p) from the Tukey's test that the means of the selected groups are identical. **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$. Serum amino acid concentrations are expressed as μM , brain amino acid content as nmol/gm brain wet weight, and brain monoamine neurotransmitters and their metabolites are expressed relative to the mean of untreated Pah-/- mice (both genders combined). The gray line labeled +/- represents the mean of wild type Pah+/+ mice (both genders combined) for comparison. (**A**) Serum phenylalanine, (**B**) brain phenylalanine, (**D**) serum tyrosine, (**E**) brain tyrosine, (**F**) relative brain dopamine, (**G**) relative brain homovanillic acid (HVA), (**H**) serum tryptophan, (**I**) brain tryptophan, (**J**) relative brain serotonin, (**K**) relative brain 5-hydroxyindoleacetic acid (5-HIAA). (**C**) Photograph of an untreated Pah-/- mouse (**Pahenu2**) and a Pah-/- mouse eight weeks after receiving rAAV2/8-mediated liver-directed gene therapy (**AAV8**) demonstrating the change in coat color associated with normalization of blood Phe concentration.

Supplementary Figure 1. Correlations between brain tryptophan, serum phenylalanine, or brain phenylalanine and the serotonin axis.



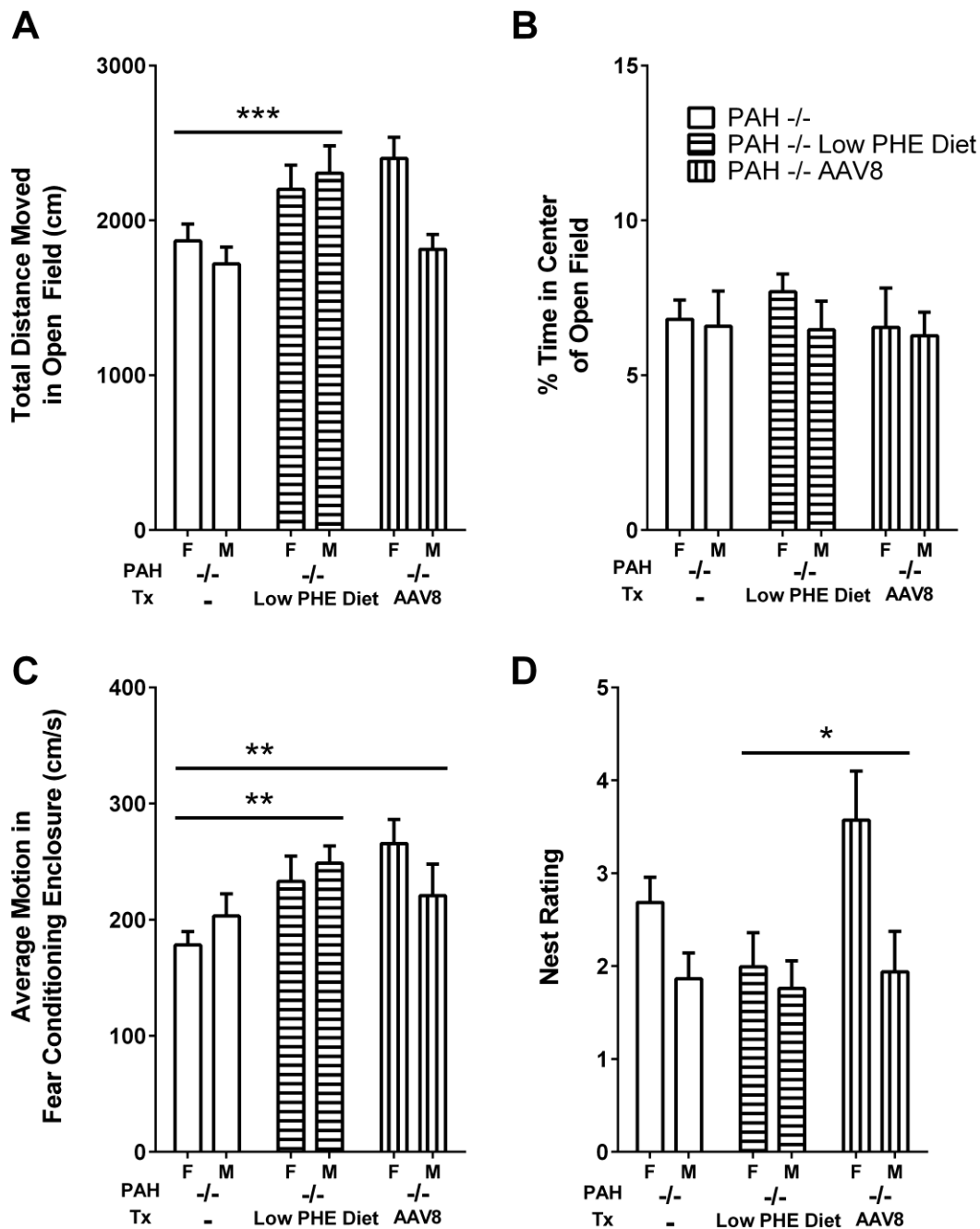
Correlations between brain serotonin or 5-HIAA (pmol/mg protein) and brain tryptophan (nmol/gm wet weight), serum phenylalanine (μ M), or brain phenylalanine (nmol/g wet weight). Linear regression analysis was carried out for each data set. Goodness of fit (r^2) and the probability (p) that the slope of the regression line equals zero are depicted on each chart. **(a)** Brain serotonin vs. brain tryptophan, **(b)** brain 5-HIAA vs. brain tryptophan, **(c)** brain serotonin vs. serum phenylalanine, **(d)** brain 5-HIAA vs. serum phenylalanine, **(e)** brain serotonin vs. brain phenylalanine, **(f)** brain 5-HIAA vs. brain phenylalanine.

3.4 Reduction of blood and brain phenylalanine is associated with improvements in open field activity and nesting behavior but little change in memory deficits of *Pah^{enu2/enu2}* mice.

Phe-reducing treatment was associated with significant improvements in open field activity and nesting behavior but had only a minor effect upon the cognitive deficits. Analysis of distance moved in the open field (**Figure 6A**) revealed an effect of treatment ($p = 0.0032$), but no effect of sex ($p = 0.1108$), and no interaction between treatment and sex. However, post hoc Tukey's multiple comparison demonstrated a significant difference only between *Pah*^{-/-} and Phe-restricted diet treated *Pah*^{-/-} mice ($p < 0.01$); a trend for a positive effect of rAAV2/8 gene therapy was only observed in females. There were no significant effects of treatment upon measures of anxiety in the open field, as measured by the % time spent in the center of the open field (**Figure 6B**). Similar differences in movement were seen in the fear conditioning enclosure (**Figure 6C**). Analysis of baseline motion, prior to any tone or shock exposure, revealed a significant treatment effect ($p = 0.0049$) but no effect of sex ($p = 0.9296$) or treatment by sex interaction. Untreated *Pah*^{-/-} mice moved less than *Pah*^{-/-} mice on low Phe diet ($p < 0.01$) and *Pah*^{-/-} receiving AAV8 ($p < 0.01$).

In *Pah*^{-/-} mice, there also was an effect of treatment on nest building ($p = 0.045$, **Figure 6D**). Nest building was more elaborate in *Pah*^{-/-} mice that received AAV8 than those that received a low Phe diet ($p = 0.035$). In *Pah*^{-/-} mice there was also an effect of sex on nest building ($p = 0.011$), with more elaborate nest building in female than male mice.

Figure 6. Effects of Phe-reducing treatments on behavioral testing in C57BL/6-*Pah*^{enu2/enu2} mice.

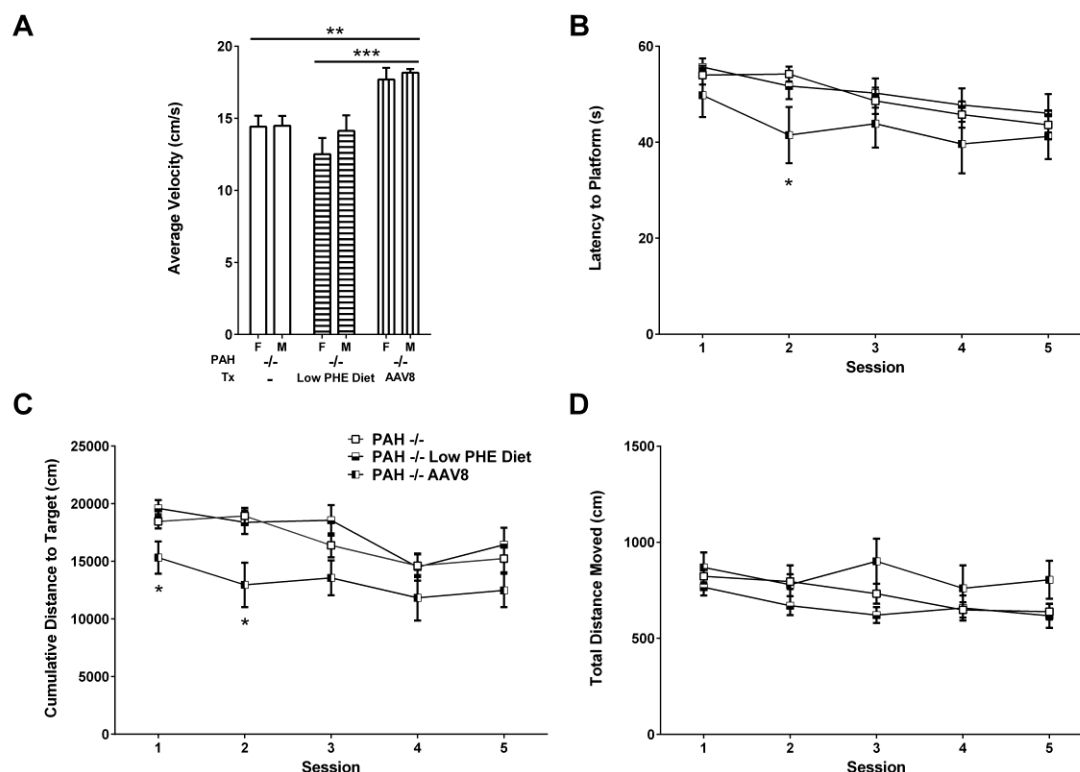


Results of behavioral performance in the open field, fear conditioning chamber, and nesting behavior of female (F) and male (M) untreated *Pah*^{-/-} mice, *Pah*^{-/-} mice receiving a phenylalanine-restricted diet (**PAH**^{-/-} low Phe diet), and *Pah*^{-/-} mice following AAV-mediated liver-directed gene therapy (**PAH**^{-/-} AAV8). The results of the

behavioral performance in the open field are presented in panel (A) activity in the open field (total distance moved) and (B) measures of anxiety in the open field (% time in center). Baseline activity while in the fear conditioning enclosure is shown in panel (C). Nest building (qualitative nest rating scale) is presented in panel (D). All data are expressed as mean \pm SEM. (A) In Pah-/- mice, higher activity levels were seen in mice on a low Phe diet compared to those on a regular diet in the open field enclosure. (B) There were no effects of treatment on measures of anxiety in the center of the open field. (C) Baseline activity in the fear conditioning enclosure showed similar treatment effects. Pah-/- mice moved less than Pah-/- mice treated with low Phe diet and Pah-/- treated with AAV8. (D) Pah-/- mice treated with AAV8 showed more complex nest building than Pah-/- mice treated with low Phe diet. Vertical bars and asterisks above the columns indicate the probability (p) from the Tukey's test that the means of the selected groups are identical. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Liver gene therapy with rAAV2/8 was associated with a small improvement in cognitive performance of Pah-/- mice as measured by ability to reach the visible platform in the water maze. Two-way ANOVA revealed effects of both treatment ($p = 0.002$) and sex ($p = 0.001$) for cumulative distance to the visible platform in the water maze (**Figure 7C**), with differences between rAAV2/8-treated and untreated Pah-/- mice ($p = 0.02$) and between rAAV2/8 treated and diet treated Pah-/- mice ($p = 0.006$) by post hoc multiple group comparisons. In addition, females showed a higher cumulative distance to the visible platform than males. Similar results were seen when latency was used as performance measures (**Figure 7B**). Trends in improved cognitive performance among diet treated Pah-/- mice were observed but did not reach statistical significance. However, these effects were not seen when total distance moved was analyzed as performance (**Figure 7D**). When average swim speeds were analyzed, there was a significant effect of genotype ($p = 0.001$), with Pah-/- treated with AAV8 swimming faster than untreated Pah-/- ($p = 0.007$) and Pah-/- diet-treated mice ($p = 0.001$). Thus, enhanced swim speeds and motor function might have contributed to the improvement in water maze performance.

Figure 7. Effects of Phe-reducing treatments on cognitive testing in C57BL/6-*Pah*^{enu2/enu2} mice.



Results of cognitive performance in the Morris water maze of female and male untreated *Pah*^{-/-} mice, *Pah*^{-/-} mice receiving a phenylalanine-restricted diet (**PAH**^{-/-} **low Phe diet**), and *Pah*^{-/-} mice following AAV-mediated liver-directed gene therapy (**PAH**^{-/-} **AAV8**). The results of water maze testing are presented in panel (**A**) average velocity (cm/second), (**B**) latency to the target platform (seconds), (**C**) cumulative distance to the target (cm), and (**D**) total distance moved (cm) with results shown for both sexes combined. (**A**) PAH^{-/-} mice that received AAV8 swam faster than both *Pah*^{-/-} untreated and *Pah*^{-/-} low Phe diet mice. (**B**) When latency to locate the platform was analyzed, there was a significant treatment effect in the second session. (**C**) *Pah*^{-/-} mice that received AAV8 showed better performance than genotype-matched mice that received no treatment ($p = 0.020$) or a low Phe diet ($p = 0.006$) and significant treatment effects were seen in the first and second sessions. (**D**) Analyzing total distance moved did not reveal any differences between the treatment groups. The data are represented as mean \pm SEM for each experimental group at each individual session. For the treatment

effects, asterisks denote sessions in which results in the treatment groups differed significantly from that of untreated *Pah*^{-/-} mice.

Cumulatively, these data argue that the behavioral phenotype of hyperphenylalaninemic mice is partly reversible even though the animals had not been treated until adulthood. Nesting behavior in mice, an indicator of health and welfare involving limbic and frontal cortical areas (Gaskill et al. 2013), may be construed as a function analogous to attention, executive functioning, and activities of daily living in humans (Deacon 2006; Torres-Lista and Gimenez-Llort 2013; Heller et al. 2014; Jirkof 2014). HPA in previously well treated adult humans with PKU is known to be associated with significant impairment of executive functioning with abundant evidence that these problems are related to concurrent HPA and are reversible with improved dietary Phe control (VanZutphen et al. 2007; Christ et al. 2010; Huijbregts et al. 2013; Jahja et al. 2013). In our experiment, improvements in nesting behavior with Phe-reducing treatment were associated with increased brain dopamine and serotonin. Administration of dopamine and serotonin reuptake inhibitors to female rats after gestation has been shown to be associated with increased frequency of nesting behavior (Johns et al. 2005). We hypothesize that increased brain monoamine neurotransmitter content in treated *Pah*^{-/-} mice was specifically responsible for the behavioral improvements, but our study cannot directly prove this hypothesis. Further experiments to specifically manipulate CNS dopamine and serotonin content without altering blood and brain Phe will be needed to critically evaluate the connection between behavioral impairments and CNS monoamine neurotransmitter deficiency in *Pah*^{enu2/enu2} mice.

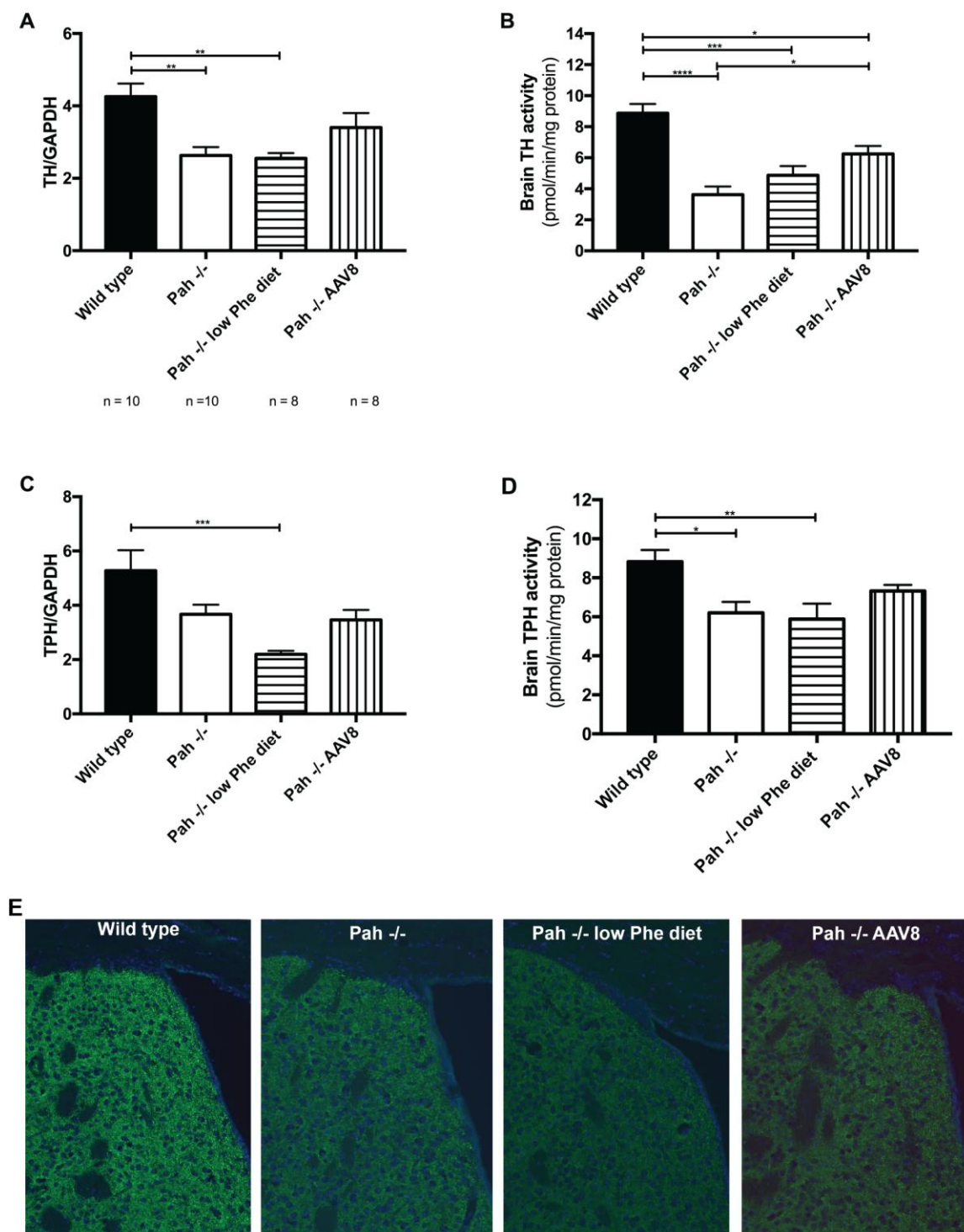
Clearly, however, significant cognitive limitations continue to exist in treated *Pah*^{-/-} mice, presumably because HPA during the juvenile period severely and permanently altered brain development. The adult *Pah*^{-/-} mice, whether treated or not, exhibit significantly decreased brain weight in comparison to *Pah*^{+/+} mice (male *Pah*^{+/+} mean brain weight = 442 ± 9 mg vs. male *Pah*^{-/-} = 391 ± 3 mg ($p < 0.0001$), female *Pah*^{+/+} = 448 ± 5 mg vs. female *Pah*^{-/-} = 378 ± 4 mg ($p < 0.0001$)). Neuropathological evaluation of the untreated adult *Pah*^{-/-} mouse brain shows disturbances of white matter integrity (Dyer et al. 1996) and decreased complexity of dendritic spine development with fewer synaptic connections but normal neuron numbers (Horling et al. 2015). These neuropathological

findings are very similar to those described in rare reports of brain histology from humans with untreated PKU (Alvord et al. 1950; Malamud 1966; Bauman and Kemper 1982). Our data strongly suggest that the severe cognitive deficits we and others (Zagreda et al. 1999; Cabib et al. 2003; Bruinenberg et al. 2016) have documented in *Pah*^{-/-} mice are likely linked to impaired neuronal development of hyperphenylalaninemic juvenile mice that is not reversed by Phe-reducing therapy initiated during adulthood.

3.6 Tyrosine hydroxylase and tryptophan hydroxylase expression is reduced in *Pah*^{enu2/enu2} brain and is not affected by Phe lowering therapies.

Correction of brain monoamine neurotransmitter content following Phe-lowering treatments in *Pah*^{-/-} mice could have been mediated by an increase in brain TH or TPH2 expression leading to improved rates of dopamine and serotonin production. We found that the abundance of TH (**Figure 8A**) and TPH2 (**Figure 8C**) proteins relative to GAPDH as assessed by Western blot was reduced in brain homogenates of *Pah*^{-/-} mice in comparison to wild type animals. Measurement of brain TH (**Figure 8B**) and TPH (**Figure 8C**) activities assayed *in vitro* under optimized and maximally stimulated conditions demonstrated that adult *Pah*^{-/-} mice express less TH and TPH2 in brain than *Pah*^{+/+} mice (see also **Supplementary Table 3**). TH activity measured in *Pah*^{-/-} mouse half brain homogenate was only 3.63 ± 0.52 pmol/min/mg protein in comparison to 8.87 ± 0.59 pmol/min/mg in wild type mice. Visualization of TH (**Figure 8E**) abundance in striatum using immunohistology confirmed this difference. TPH2 activity was similarly reduced in *Pah*^{-/-} mice (*Pah*^{-/-} = 6.20 ± 0.56 pmol/min/mg; wild type = 8.82 ± 0.60 pmol/min/mg). Importantly, reduction of blood and brain Phe, whether through dietary Phe restriction or liver-directed gene therapy, was not associated with any significant change in brain TH or TPH protein content or maximal activity in *Pah*^{-/-} mice. Therefore, the corrections in brain monoamine content we measured following Phe-reducing therapies were not caused by increased production of TH and TPH protein.

Figure 8. Expression of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH2) in brain of C57BL/6-*Pah*^{enu2/enu2} mice and effects of phenylalanine reducing treatments.



The abundance of **(A)** TH and **(C)** TPH2 proteins in half brain homogenates from wild type mice (5 male, 5 female), untreated *Pah*^{-/-} mice (5 male, 5 female), and *Pah*^{-/-} mice

(4 male and 4 female in each group) treated with either dietary phenylalanine restriction or AAV8 liver gene therapy were measured relative to GAPDH by Western blotting. The data are presented as the mean \pm SEM TH/GAPDH or TPH/GAPDH ratios. The maximally stimulated TH and TPH activities (panels **(B)** and **(D)** respectively) in half brain homogenates were measured in vitro and the data were expressed as mean \pm SEM pmol reaction product produced/min/mg brain protein. Differences across groups were explored using one-way ANOVA with post hoc Holm-Sidak's multiple comparisons test. Vertical bars and asterisks above the columns indicate the probability (p) from the Tukey's test that the means of the selected groups are identical. **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$.

TH (panel **E**, 200 X magnification) abundance in microscopic sections of brain striatum was detected by immunohistology and fluorescence microscopy (representative images shown).

4. DISCUSSION

4.1 Phe-mediated inhibition of TH and TPH activities is predominantly responsible for monoamine neurotransmitter deficiencies in *Pah^{enu2/enu2}* mouse brain.

Given the extreme HPA in *Pah*^{-/-} mice and putative saturation of the LAT1 transporter, we had expected to measure severely decreased brain Tyr and Trp content. However, In the experiments reported here and in our previous work (Harding et al. 2014), brain Tyr and Trp contents were only minimally reduced in *Pah*^{-/-} mice in comparison to *Pah*^{+/+} mice, even though the blood concentrations of these amino acids were low. Also, brain Tyr and Trp contents were not substantially altered by Phe-reducing treatment. A separate, sodium dependent, energy requiring LNAA transporter with even higher affinity ($K_m \sim 0.2 \mu M$) but lower transport capacity than LAT1 is expressed on the abluminal side of the BBB (O'Kane and Hawkins 2003). This system is capable of transporting LNAA from ECF to the circulation against the gradient. The two transport systems together insure a constant LNAA supply to the brain but with homeostatic control of LNAA flux to maintain constant LNAA concentrations in the brain independent of any variation in blood LNAA levels (Hawkins et al. 2006). We propose that this homeostatic mechanism is capable of maintaining brain Tyr and Trp content despite extremely increased blood and brain Phe content in PKU.

In our experiments, values of brain dopamine and serotonin in our experiment correlated strongly with either blood or brain Phe content. These data are consistent with a previous report that brain serotonin measured through microdialysis of the *Pah*^{-/-} mouse prefrontal cortex were more consistently related to brain Phe than brain Trp (Pascucci et al. 2008). Phe-reducing treatment, either dietary Phe restriction or rAAV2/8-mediated liver-directed gene therapy, in our hands was associated with near normalization of brain monoamine neurotransmitter content and turnover in *Pah*^{-/-} mice. Furthermore, correction of monoamine content was more complete in diet-treated mice that exhibited lower blood Phe than in rAAV2/8-treated mice with a greater range of blood Phe concentrations, despite the fact that diet-treated mice exhibited persistent blood Tyr deficiency. We have also shown here that the abundance of TH and TPH protein is constitutively reduced in *Pah*^{-/-} brain and is unaltered by Phe-reducing therapies initiated during adulthood. Additionally, TH activity measured in vivo through the analysis of L-DOPA production in microdialysates obtained from *Pah*^{-/-} mouse prefrontal cortex was

strongly inhibited in hyperphenylalaninemic mice (Pascucci et al. 2012). Taken together, our results along with published data lend greater support to the hypothesis that monoamine neurotransmitter deficiency in PKU is predominantly caused by a reduction in TH and TPH2 enzymatic levels and Phe-mediated inhibition of both activities rather than limited substrate availability.

4.2 Implications for Large Neutral Amino Acid (LNAA) supplementation as therapy for PAH deficiency.

If Phe-mediated inhibition of TH and TPH2 activities is the predominant mechanism causing monoamine neurotransmitter deficiency in PKU, then Tyr and Trp supplementation alone, without concomitant reductions in blood and brain Phe, will not substantially ameliorate CNS monoamine neurotransmitter deficiency in hyperphenylalaninemic mice or humans. Dietary Tyr supplementation without dietary Phe restriction has been proposed as a potential therapeutic alternative for PKU, although interestingly little attention has been paid in the existing literature to the possible effects of Trp deficiency. Tyr supplementation as sole therapy for infants with PKU without reduction of blood Phe does not prevent severe cognitive disability (Batshaw et al. 1981), but oral Tyr supplementation in hyperphenylalaninemic adults, some of whom had been late-treated, has been associated with increased cerebrospinal fluid (CSF) neurotransmitter levels and improved visual reaction times (Lou et al. 1987; Lykkelund et al. 1988). Other investigators however have found no consistent benefit of Tyr supplementation among early-treated adults in the context of poor dietary Phe control (Pietz et al. 1995).

Oral supplementation with a mixture of LNAA including Tyr and Trp but without dietary Phe restriction has been widely advocated as a possible means of preventing Phe-mediated CNS effects in hyperphenylalaninemic adults with the rationale that LNAA would successfully compete against Phe for uptake into brain, lead to lower brain Phe content, and restore any CNS metabolic disturbance associated with increased brain Phe content (reviewed in (Rocha and Martel 2009)). Several different LNAA mixtures formulated as powders or tablets are commercially available for oral treatment of adults with PKU. Pietz and colleagues reported that cerebral Phe content as measured by proton magnetic resonance spectroscopy following an oral Phe challenge in six adults with PKU was significantly reduced by oral supplementation with a mixture of valine,

leucine, isoleucine, methionine, tyrosine, histidine, and tryptophan (Pietz et al. 1999). A subsequent open label clinical trial of this LNAA mixture plus added histidine and arginine was associated with increased blood Tyr and Trp concentration but no effect upon blood Phe (Koch et al. 2003). The results of our experiment reported here predict little effect upon brain Phe, Tyr, Trp, or monoamine neurotransmitters following administration of this LNAA mixture if blood Phe were not altered. However, Schindeler and colleagues have demonstrated a positive benefit upon executive function in hyperphenylalaninemic adults treated with LNAA in an open label clinical trial even if blood Phe was not altered by the therapy (Schindeler et al. 2007). Recent investigations in the *Pah^{enu2}* mouse suggest that an appropriately designed LNAA mixture could improve CNS monoamine neurotransmitter content through three potential mechanisms: reduction of brain Phe through competition for transport at the BBB, increased brain Tyr and Trp content, and finally reduced inhibition of TH and TPH2 activities (van Vliet et al. 2015; van Vliet et al. 2016). Careful review of those data reveal that LNAA treatment was associated with significant reductions in both blood and brain Phe. Given the results of our experiment reported here, we hypothesize that LNAA-mediated reduction in blood Phe and consequently reduced brain Phe likely was predominantly responsible for the improved brain serotonin content in LNAA-treated *Pah^{enu2}* mice.

5. CONCLUSION

We report that Phe-reducing treatment, either dietary Phe restriction or rAAV2/8-mediated liver-directed gene therapy, was associated with significantly improved CNS neurotransmitter status in *Pah^{enu2/enu2}* mice. These data are consistent with a previous report of improved brain neurotransmitters following liver-directed gene therapy with an adeno-associated virus vector in BTBR-*Pah^{enu2/enu2}* mice (Yagi et al. 2012) and a report of improved behavior in BTBR-*Pah^{enu2/enu2}* mice following treatment with a PAH-expressing helper dependent adenoviral vector (Cerreto et al. 2012). Improvements in brain neurotransmitter content, predominantly of the serotonin axis, correlated primarily with serum and brain Phe concentrations and not the brain concentrations of either Tyr or Trp, the precursors of dopamine and serotonin respectively. The data suggest that Phe-mediated competitive inhibition of TH and TPH2 activities is the predominant mechanism behind monoamine neurotransmitter deficiency in hyperphenylalaninemic mice. The observed biochemical improvements in *Pah*^{-/-} mice following Phe-reducing therapy initiated at 8 weeks age were associated with improved open field activity and

nesting behaviors but without meaningful change in the severe cognitive deficit exhibited by the mice. Importantly, there were no genotype differences in swim speeds, excluding the possibility that genotype differences in motor function might have contributed to genotype difference in performance in the water maze. Prevention of these cognitive deficits likely requires initiation of Phe-reducing therapy during early infancy to allow normal brain development. Indeed, liver-directed gene therapy administered at 3 weeks age has been associated with improved performance in the water maze by BTBR-*Pah*^{enu2/enu2} mice (Cerreto et al. 2012). Although the improvements in activity levels in the open field and nesting behavior in our experiment were associated with significantly increased brain serotonin content, further experiments will be necessary to investigate the specific hypothesis that serotonin deficiency is the proximal molecular mechanism causing altered behavioral performance and impaired cognitive function in hyperphenylalaninemic *Pah*^{enu2/enu2} mice.

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SUPPLEMENTARY TABLE 1 - Genotype effects

Analyte	Pah+/+		Pah+/-		Pah-/-		Interaction		Genotype		Sex	
	Mean \pm SE		Mean \pm SE		Mean \pm SE		(F)	p	(F)	p	(F)	p
	F N = 11	M N = 8	F N = 4	M N = 4	F N = 16	M N = 11						
Serum phenylalanine (μ M)	111 \pm 11	132 \pm 16	72 \pm 7	98 \pm 23	2654 \pm 87	2198 \pm 71	7.651	0.0013	715.9	< 0.0001	4.236	0.0451
Brain phenylalanine (nmol/gm)	85 \pm 15	110 \pm 28	171 \pm 7	194 \pm 6	811 \pm 17	713 \pm 18	6.885	0.0024	753.8	< 0.0001	0.8252	0.3682
Serum tyrosine (μ M)	91 \pm 4	72 \pm 4	57 \pm 5	57 \pm 4	44 \pm 3	36 \pm 3	2.126	0.1307	61.61	< 0.0001	5.495	0.0234
Brain tyrosine (nmol/gm)	88 \pm 11	101 \pm 22	163 \pm 20	173 \pm 18	91 \pm 13	79 \pm 18	0.3774	0.6877	8.131	0.0009	0.05591	0.8141
Serum tryptophan (μ M)	118 \pm 5	69 \pm 3	89 \pm 3	82 \pm 3	78 \pm 3	68 \pm 2	18.94	< 0.0001	17.22	< 0.0001	43.49	< 0.0001
Brain tryptophan (nmol/gm)	19 \pm 2	20 \pm 4	24 \pm 2	25 \pm 1	17 \pm 2	14 \pm 2	0.5508	0.5802	4.248	0.0201	0.00144	0.9699
Dopamine (pmol/mg protein)	201 \pm 15	203 \pm 21	350 \pm 54	316 \pm 39	156 \pm 16	210 \pm 19	1.729	0.1884	16.29	< 0.0001	0.1226	0.7278
Homovanillic acid (pmol/mg protein)	29 \pm 1.3	26 \pm 2.1	49 \pm 4.1	40 \pm 3.2	21 \pm 1.7	28 \pm 2.1	4.502	0.0161	33.89	< 0.0001	1.081	0.3037
Serotonin (pmol/mg protein)	80 \pm 2.9	75 \pm 6.2	166 \pm 4.7	148 \pm 12	38 \pm 2.4	43 \pm 5.3	2.090	0.1348	210.7	< 0.0001	1.695	0.1991
5-HIAA	73 \pm 5.5	51 \pm 2.3	159 \pm 5.5	132 \pm 12	17 \pm 1.6	22 \pm 3.3	8.439	0.0007	321.7	<	14.92	0.0003

(pmol/mg protein)										0.0001		
Normalized neurotransmitters												
Dopamine (ratio to mean Pah-/-)	1.44 ± 0.11	1.39 ± 0.15	1.37 ± 0.21	1.23 ± 0.15	0.92 ± 0.07	1.15 ± 0.07	1.477	0.2385	7.544	0.0014	0.0289	0.8658
Homovanillic acid (ratio to mean Pah-/-)	1.53 ± 0.07	1.42 ± 0.12	1.57 ± 0.13	1.28 ± 0.10	0.93 ± 0.04	1.12 ± 0.06	4.642	0.0144	24.21	< 0.0001	1.039	0.3132
Serotonin (ratio to mean Pah-/-)	2.31 ± 0.09	2.10 ± 0.17	3.00 ± 0.18	2.67 ± 0.22	0.97 ± 0.05	1.10 ± 0.07	2.636	0.0820	148.8	< 0.0001	2.059	0.1578
5-HIAA (ratio to mean Pah-/-)	4.93 ± 0.29	3.74 ± 0.20	5.36 ± 0.19	4.45 ± 0.41	0.95 ± 0.06	1.08 ± 0.03	8.326	0.0008	264.7	< 0.0001	14.63	0.0004

P values < 0.05 are highlighted in light blue.

SUPPLEMENTARY TABLE 2 - Treatment effects

Analyte	Pah-/- Mean ± SE		Low Phe diet Mean ± SE		AAV8 Mean ± SE		Interaction		Treatment		Sex	
	F N = 16	M N = 11	F N = 9	M N = 9	F N = 7	M N = 4	(F)	p	(F)	p	(F)	p
Serum phenylalanine (μM)	2654 ± 87	2198 ± 71	494 ± 111	295 ± 67	961 ± 220	407 ± 175	1.066	0.3525	198.2	< 0.0001	15.44	0.0003
Brain phenylalanine (nmol/gm)	811 ± 17	713 ± 18	326 ± 41	244 ± 17	408 ± 72	206 ± 41	1.346	0.27	151.9	< 0.0001	19.42	< 0.0001
Serum tyrosine (μM)	44 ± 3	36 ± 3	32 ± 2	31 ± 3	86 ± 10	98 ± 9	1.79	0.1779	65.95	< 0.0001	0.0618	0.8047
Brain tyrosine	91 ± 13	79 ± 18	100 ± 24	80 ± 19	102 ± 15	93 ± 20	0.0384	0.9623	0.1957	0.8229	0.699	0.4073

(nmol/gm)												
Serum tryptophan (μ M)	78 \pm 3	68 \pm 2	107 \pm 5	86 \pm 3	100 \pm 3	86 \pm 3	1.629	0.2068	27.25	< 0.0001	23.24	< 0.0001
Brain tryptophan (nmol/gm)	17 \pm 2	14 \pm 2	23 \pm 2	19 \pm 2	20 \pm 2	16 \pm 3	0.0099	0.9901	3.183	0.0503	2.854	0.0976
Dopamine (pmol/mg protein)	156 \pm 16	210 \pm 19	324 \pm 79	227 \pm 17	171 \pm 15	175 \pm 8.2	2.842	0.0682	5.043	0.0103	0.1940	0.6616
Homovanillic acid (pmol/mg protein)	21 \pm 1.7	28 \pm 2.1	38 \pm 9.0	28 \pm 2.1	24 \pm 2.5	23 \pm 1.7	2.138	0.1289	4.142	0.0219	0.2882	0.5939
Serotonin (pmol/mg protein)	38 \pm 2.4	43 \pm 5.3	151 \pm 28	110 \pm 7.2	66 \pm 15	60 \pm 3.3	2.051	0.1398	32.42	< 0.0001	1.749	0.1922
5-HIAA (pmol/mg protein)	17 \pm 1.6	22 \pm 3.3	124 \pm 30	87 \pm 12	36 \pm 6.2	34 \pm 2.5	1.74	0.1864	29.81	< 0.0001	1.195	0.2798
Normalized neurotransmitters												
Dopamine (ratio to mean Pah-/-)	0.92 \pm 0.07	1.15 \pm 0.07	1.56 \pm 0.29	1.14 \pm 0.07	1.29 \pm 0.11	1.32 \pm 0.06	3.621	0.0343	3.784	0.0298	0.2259	0.6368
Homovanillic acid (ratio to mean Pah-/-)	0.93 \pm 0.04	1.12 \pm 0.06	1.49 \pm 0.71	1.17 \pm 0.07	1.27 \pm 0.13	1.21 \pm 0.09	2.938	0.0626	4.383	0.0179	0.3862	0.5372
Serotonin (ratio to mean Pah-/-)	0.97 \pm 0.05	1.10 \pm 0.07	3.11 \pm 0.41	2.39 \pm 0.12	2.01 \pm 0.46	1.82 \pm 0.10	2.143	0.1284	35.40	< 0.0001	1.844	0.1808
5-HIAA (ratio to mean Pah-/-)	0.95 \pm 0.06	1.08 \pm 0.03	5.59 \pm 0.69	4.28 \pm 0.32	2.25 \pm 0.38	2.11 \pm 0.16	3.156	0.0516	92.38	< 0.0001	2.587	0.1143

P values < 0.05 are highlighted in light blue.

SUPPLEMENTARY TABLE 3

Brain tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH2) content

Analyte	Wild type n = 10	PAH -/- n = 10	Low Phe diet n = 8	AAV8 n = 8	One way ANOVA	
	Mean \pm SE	Mean \pm SE	Mean \pm SE	Mean \pm SE	F	p
TH/GAPDH ratio	4.26 \pm 0.35	2.63 \pm 0.23	2.55 \pm 0.15	3.40 \pm 0.40	7.265	0.0008
TH activity (pmol/min/mg)	8.87 \pm 0.59	3.63 \pm 0.52	4.87 \pm 0.60	6.24 \pm 0.52	17.45	<0.0001
TPH2/GAPDH ratio	5.28 \pm 0.76	3.67 \pm 0.36	2.19 \pm 0.37	3.46 \pm 0.37	6.487	0.0015
TPH activity (pmol/min/mg)	8.82 \pm 0.60	6.20 \pm 0.56	5.88 \pm 0.79	7.32 \pm 0.32	5.249	0.0046

TH and TPH2 protein abundance in half brain homogenates (equal numbers of males and females in each group) were analyzed by immunoblotting and expressed relative to GAPDH. Maximally stimulated TH and TPH activities were measured in half brain homogenates as previously described and expressed as pmol product/min/mg brain protein. Differences among the four groups in each assay were explored using one-way analysis of variance (ANOVA) followed by post hoc intergroup comparisons with Holm-Sidak's multiple comparisons test.

Highlights

- Chronic hyperphenylalaninemia in C57Bl6-*Pah*^{enu2/enu2}, a model of human phenylketonuria, is associated with brain dopamine and serotonin deficiencies with severe behavioral alterations and cognitive impairment.
- Phenylalanine reducing treatments, including dietary phenylalanine restriction or liver directed gene therapy, initiated during adulthood yielded increased brain monoamine neurotransmitter content and improved nesting behavior, but no change in cognitive impairments.
- Phenylalanine-mediated competitive inhibition of tyrosine hydroxylase and tryptophan hydroxylase 2 activities appears to be the predominant mechanism causing CNS monoamine neurotransmitter deficiency in hyperphenylalaninemic mice.